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Cooperative and Harmful Behaviour in the Bacterial World

Doutoramento em Biologia

Especialidade em Biologia Evolutiva

Iolanda Lopes Domingues

Tese orientada por:

Professor Doutor Francisco Dionísio

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Documento especialmente elaborado para a obtenção do grau de doutor

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Júri:

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- Doutor Francisco André de Campos Pereira Dionísio

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SUMMARY

Bacteria are social organisms capable of displaying a multiplicity of complex behaviours, some of them with a significant impact on human lives. Antibiotic resistance, for one, is currently a major health menace and is typically envisioned as an asocial behaviour. Yet, sensitive bacteria can survive the action of antibiotics, given that their social entourage gathers the right characteristics. In this thesis social behaviour of bacteria, ranging from altruistic to spiteful, are shown to affect not only their survival, but also their ability to counterattack the invasion of competing bacteria, and ensure the preservation of social traits, such as antibiotic resistance.

To ascertain the complexity and relevance of social behaviours on the bacterial world we studied two types of *Escherichia coli* mobile genetic elements: bacteriophages and plasmids. Such elements, are not only able to transmit horizontally between different bacterial lineages, but are also able to promote social behaviour in bacteria. In this thesis, both a temperate bacteriophage and three different conjugative plasmids were shown to act as promotors of bacterial social behaviours – both cooperative and harmful.

Lysogenic bacteria were shown to use the λ bacteriophage as an allelopathic agent able to harm susceptible cells in their vicinity. This behaviour is of a spiteful nature towards the killed susceptible cells, but also proves to be altruistic towards surviving lysogenic bacteria in the population. Similarly, ampicillin-resistant bacteria, carrying conjugative plasmids, were able to cooperate in the detoxification of ampicillin enriched environments, which led to the survival of genetically sensitive bacteria. However, such sensitive hitchhikers did not remain unharmed for long. In fact, the resistant bacteria were able to use plasmids as a mechanism to harm plasmid-free bacteria and also to restore the cooperative antibiotic-resistance in the population.

There is a great need to increase the general knowledge about bacterial social behaviours, since they are involved in well-known threats to public's health. As far as bacteria are concerned, especially pathogenic bacteria, it is urgent to understand how

social behaviours influence the ability of strains to survive the action of antibiotics, but also how they are able to cope when competing against non-pathogenic strains.

Key Words

Plasmid; Bacteriophage; Antibiotic Resistance; Altruism; Spite

RESUMO

As bactérias são organismos sociais capazes de desempenhar uma multiplicidade de comportamentos complexos, alguns dos quais com um impacto significativo na vida dos seres humanos. A resistência a antibióticos, por exemplo, é uma das maiores ameaças à saúde pública da atualidade e é tipicamente vista como um comportamento associal. Porém, bactérias sensíveis podem sobreviver à ação de antibióticos, se o seu enquadramento social reunir as características necessárias. Nesta tese, mostra-se que comportamentos sociais bacterianos, desde altruísmo a malícia, são capazes de afetar não só a sua sobrevivência como também a sua habilidade de contra-atacar a invasão de bactérias competidoras, assegurando a preservação de traços sociais, tais como a resistência a antibiótico.

Por forma a desenvolver os conhecimentos relativos à complexidade e relevância dos comportamentos sociais no "mundo bacteriano", estudamos dois tipos de elementos genéticos móveis de *Escherchia coli*: bacteriófagos e plasmídeos. Esses elementos são, não só capazes de ser transferidos horizontalmente entre diferentes linhagens bacterianas, como também são capazes de promover comportamentos sociais em populações bacterianas. Nesta tese, demonstra-se que, tanto um bacteriófago temperado como três plasmídeos conjugativos, atuam como promotores de comportamentos sociais – sejam cooperativos ou prejudiciais.

É demonstrado que bactérias lisogénicas podem usar o fago λ como um agente alelopático capaz de prejudicar células suscetíveis na sua vizinhança. Este comportamento é de uma natureza maliciosa do ponto de vista das bactérias suscetíveis, mas também se mostra altruístico para com as outras bactérias lisogénicas que existem na população. Da mesma forma que bactérias resistentes a ampicilina, que possuem plasmídeos conjugativos, foram capazes de cooperar na destoxificação de um ambiente suplementado com ampicilina, o que por sua vez levou à sobrevivência de bactérias geneticamente sensíveis à ampicilina. No entanto, essas bactérias sensíveis oportunistas não permaneceram impunes por muito tempo. De facto, as bactérias resistentes foram capazes de usar os plasmídeos como uma forma de prejudicar as

bactérias inicialmente sem plasmídeo e também como forma de restaurar o comportamento cooperativo de resistência a antibióticos na população.

Existe uma grande necessidade em aumentar o conhecimento geral acerca de comportamentos sociais bacterianos, uma vez que estes organismos estão envolvidos em ameaças à saúde pública bem conhecidos. Em relação a bactérias, especialmente bactérias patogénicas, é urgente perceber como é que comportamentos sociais influenciam a capacidade de sobrevivência de estirpes à ação de antibióticos, mas também como é que elas essas estirpes são capazes de lidar quando em competição com bactérias não patogénicas.

Palavras-Chave:

Plasmídeo, Bacteriófago, Resistência Antibiótica, Altruísmo, Malícia

RESUMO ALARGADO

Nas últimas décadas, tem ocorrido uma crescente consciencialização de que, ao contrário da ideia tradicional, as bactérias são seres capazes de exibir diversos comportamentos sociais. Isto é, uma bactéria pode exibir um comportamento que vai surtir efeito noutras bactérias na sua vizinhança populacional.

Os comportamentos sociais bacterianos são um fenómeno com interesse próprio pois ajudam-nos a compreender a evolução do comportamento social de uma forma geral bem como certos aspetos da ecologia ou mesmo da virulência bacteriana. No entanto, estudar o comportamento social entre bactérias também se reveste de alguma importância pois pode ter um impacto significativo na sociedade humana, tanto positiva como negativamente. Um bom exemplo de um comportamento social com um impacto negativo para os humanos é a emergência de resistência a antibióticos que é, neste momento, uma grande ameaça à saúde pública, e é para além disso, conservadoramente, encarada como um comportamento associal. No entanto, alguns estudos recentes demonstraram o contrário. A suscetibilidade das bactérias à ação de um antibiótico pode ser ajustada de acordo com o seu enquadramento social. Foi previamente demonstrada a capacidade de células sensíveis a antibióticos β-lactâmicos de sobreviver na presença de antibióticos dessa classe, quando na presença de células resistentes. Estas observações foram obtidas, por exemplo, através da utilização de bactérias em que o gene de resistência (β-lacatamse) se encontrava codificado num plasmídeo artificial. Nesses trabalhos, a produção de β-lacatamse foi otimizada, o que resultou na proteção de estirpes sensíveis que se encontravam na presença de células produtoras de β-lacatamse. Estes trabalhos demonstraram a capacidade de bactérias resistentes protegerem células da mesma espécie (Dugatkin, Perlin et al. 2005, Clark, Alton et al. 2009) ou de uma espécie diferente (Perlin, Clark et al. 2009). A capacidade de bactérias sensíveis resistirem à ação de β-lactâmicos também foi observada *in vivo* em infeções poli-microbianas (Brook 2004).

Apesar de extremamente importantes e relevantes, os resultados que se conhecem relativamente à sobrevivência de bactérias sensíveis à ação de antibióticos, devido à

atividade de células resistentes, baseiam-se quase inteiramente em sistemas artificiais onde existe uma sobreprodução do fator de resistência em foco. A capacidade de proteção de bactérias cuja produção de β-lactamase fosse natural é, do nosso entender, extremamente relevante, e infelizmente ainda pouco foi feito nesse sentido. Por exemplo, em estudos *in vivo* (Brook 2004), a proteção de estirpes sensíveis é levada a cabo por bactérias "naturais", no entanto, as variáveis experimentais não são controladas. Os resultados apresentados no âmbito desta tese foram obtidos juntando a utilização de plasmídeos de origem natural (portanto com uma produção natural de β-lactamase) e em combinações de condições experimentais bem definidas, nomeadamente, a combinação de diferentes estruturas de habitats, e de densidade e frequência iniciais de células.

No capítulo III, mostramos que estirpes de E*scherichia coli* que possuem um de três plasmídeos conjugativos naturais (R1, R16a ou RP4) são de facto capazes de proteger uma estirpe sensível, da mesma espécie, em ambientes com ampicilina (antibiótico β-lactâmico). Este estudo foi realizado numa combinação controlada e sistemática de quatro condições diferentes: i) três plasmídeos diferentes; ii) duas estruturas de habitat (estruturado e não-estruturado); iii) três densidades iniciais (alta, intermédia e baixa); e iv) três frequências iniciais (alta frequência de sensíveis, intermédia e baixa). Resultando num total de 54 condições. Foi observada a sobrevivência da estirpe sensível em 63% dessas condições. Também foi analisada a importância das diferentes condições na capacidade de proteção, tendo sido observado que a condição mais relevante é a densidade, seguida da estrutura e finalmente o tipo de plasmídeo. Foi determinado que a combinação de condições explica 50% da variância dos resultados, e que a interação de fatores também afeta significativamente a sobrevivência das sensíveis. Ou seja, a frequência inicial, por exemplo, afeta a sobrevivência das sensíveis de forma diferente dependendo de se a densidade inicial era alta ou baixa. Quando a densidade inicial é alta, as bactérias sensíveis sobrevivem mais quando inicialmente menos frequentes. Já a baixa densidade, as bactérias sensíveis sobrevivem mais quando inicialmente mais frequentes.

No capítulo V, estuda-se com mais detalhe a protecção em baixa densidade, e mostra-se que as bactérias sensíveis sobrevivem mais quando são frequentes devido a

uma combinação de fatores. Neste caso, a sobrevivência das sensíveis após 24 horas de competição com bactérias resistentes resulta, em ultima instância, da proteção das resistentes. No entanto, a baixa densidade, as células sensíveis só chegam a ser protegidas pela β-lactamase quando, na fase inicial de contacto com a ampicilina, eram muitas. Assim, argumenta-se que este fenómeno se deve a um altruísmo "forçado" por parte das muitas sensíveis que morrem, servindo de "sorvedor" do antibiótico. Ou seja, grande parte das moléculas de ampicilina ligam-se à parece celular, ficando aí mesmo após a morte dessas células. Desta forma, muitas células sensíveis têm um papel de "sorvedores" da ampicilina presente no meio, possibilitando que algumas células que, nesse momento não se estavam a reproduzir, sobrevivam durante mais alguns minutos. Mais tarde, são protegidas pela ação da β-lactamase (quando esta atinge um nível suficiente para destoxificar o meio) produzida pelas células resistentes.

A resistência a antibióticos é um dos muitos comportamentos sociais bacterianos que se conhecem à data. No entanto, quer para bactérias quer para organismos multicelulares, a existência de comportamentos sociais como o altruísmo e a malícia é intrigante, uma vez que os atores destes comportamentos pagam um custo associado ao seu comportamento (West, Griffin et al. 2006, Diggle 2010). A Selecção Natural diz-nos que os indivíduos que hoje observamos são descendentes dos que deixaram maior prole no passado. Sendo assim, como explicar que certos indivíduos tenham comportamentos custosos, isto é, que baixem o seu sucesso reprodutivo (fitness)? Assim, o que garante a sua existência e manutenção nas populações? A resposta para a manutenção de comportamentos altruístas foi dada por Hamilton (Hamilton 1964). Um comportamento pode ser mantido, apesar de custoso para o ator, se aumentar a representação dos genes do ator na população. Ou seja, se o comportamento beneficiar indivíduos aparentados (Smith and Wynneedwards 1964).

No entanto, se uma população constituída por indivíduos altruístas for invadida por indivíduos capazes de usufruir do comportamento sem pagar o custo associado, os chamados "*cheaters*" (batoteiros), a população de altruístas poderá ver-se em dificuldades. Dado que os "*cheaters*" têm benefício sem qualquer custo, poderão reproduzir-se mais rapidamente que os indivíduos altruísmos, e neste caso, o comportamento estaria em risco de se extinguir (West, Griffin et al. 2006, Diggle 2010).

As populações bacterianas, por exemplo, cooperam frequentemente através da produção e secreção de "bens-públicos", como a β-lactamase. Ou seja, no caso de bactérias sensíveis que são protegidas pela ação de bactérias resistentes, o que está na realidade a acontecer é que as bactérias sensíveis estão a explorar a cooperação das células resistentes.

No mundo bacteriano, observa-se frequentemente a transferência horizontal de genes. Assim, Smith (2001) formulou a interessante hipótese de que as bactérias podem socorrer-se da transferência horizontal de genes em bactérias para forçar que outras bactérias cooperem (Smith 2001). Se o gene para o comportamento de cooperação estiver codificado num elemento genético móvel, as bactérias cooperadoras poderiam transferir esse gene para os "*cheaters*" obrigando-os a tornar-se cooperadores.

Resultados experimentais sugerem que este reforço, através da transferência horizontal de genes (concretamente através de conjugação) existe mas é de curta duração (em habitats não-estruturados) (Dimitriu, Lotton et al. 2014). O referido trabalho, separa o comportamento de cooperação (e a sua exploração) da conjugação, temporalmente, numa tentativa de isolar o efeito de cada componente. Nesta tese, perguntamos: o que acontece quando se inibe a conjugação, mas bactérias produtoras de β-lactamase continuam a produzir este "bem-público"? Ou seja, o que acontece às bactérias sensíveis se forem capazes de usufruir da destoxificação do meio, pela β-lactamase, mas não receber o gene através de conjugação?

Os resultados apresentados no Capítulo IV respondem a essa pergunta. Nesse capítulo, usando as mesmas estirpes do que no capítulo III, demonstra-se que ao inibir a conjugação, a estirpe sensível é protegida, ainda mais, do que quando a conjugação ocorre. A conjugação foi inibida de forma mecânica, usando membranas porosas para separar o crescimento das bactérias sensíveis e resistentes, desta forma, as células não contactam (não ocorre conjugação) mas a β-lactamase difunde-se livremente pelo meio de crescimento. Com este sistema observamos que, quando a conjugação ocorre (na presença de ampicilina), as bactérias sensíveis são prejudicadas em cerca de 88% das condições, sendo que de facto em metade dessas condições as bactérias sensíveis recebem os plasmídeos e são forçadas a tornar-se produtoras de β-lactamase, tal como previsto por Smith (2001) e demonstrado experimentalmente por Dimitriu (2014).

No entanto, também observamos que na outra metade dessas condições as bactérias cresceram menos quando em contacto com as resistentes (do que quando separadas fisicamente) mas não receberam os plasmídeos. Estes resultados mostram que para além de a conjugação poder ser um mecanismo de reforço da cooperação – através da introdução do gene para esse comportamento social nas bactérias "*cheaters*" - esta também é um mecanismo usado para castigar os "*cheaters*", impedindo-os de se reproduzir. Este comportamento é malicioso e foi, do nosso conhecimento, observado pela primeira vez neste trabalho, demonstrando que os plasmídeos bacterianos podem ser usados pelas bactérias hospedeiras como "armas biológicas", prejudicando bactérias na vizinhança.

A possibilidade de bactérias usarem os seus parasitas (como plasmídeos e bacteriófagos) como "armas biológicas" foi formulada para demonstrar que os comportamentos maliciosos podem ocorrer mais vezes na natureza do que se pensa (Dionisio 2007). Isto porque, uma vez que os comportamentos maliciosos são prejudiciais quer para o actor quer para o recipiente do comportamento, muitos autores defendem que o comportamento não pode existir. No entanto, nesta tese são demonstrados dois comportamentos de malícia, ambos envolvendo bactérias e os seus parasitas – plasmídeos (Capítulo IV) e bacteriófagos (Capítulo II).

No capítulo II, demonstra-se, usando bactérias lisogénicas (células em que o fago está integrado) da espécie *Escherichia coli,* que essas bactérias são capazes de usar o bacteriófago λ para prejudicar células sensíveis na sua vizinhança. O comportamento é considerado malicioso porque as células lisogénicas têm invariavelmente de morrer para poder libertar a descendência fágica. Este estudo foi feito em duas estruturas de habitat diferentes (estruturado e não estruturado) e em diferentes frequências de bactérias lisogénicas e suscetíveis. Foi demonstrado que em meio não estruturado, as células lisogénicas são desfavorecidas, independentemente, da frequência inicial. Mas em meio estruturado, as células lisogénicas tem vantagem em todas a frequências iniciais. Assim, mostra-se que o bacteriófago λ pode ser usado como "arma biológica" contra células da vizinhança.

Concluindo, nesta tese mostramos que o comportamento social é ubíquo em populações bacterianas, podendo mesmo ter impacto na saúde humana. No entanto, se

por um lado observámos que, facilmente, as bactérias ajudam outras a sobreviver perante situações adversas incluindo a antibióticos, também observámos que usam os seus elementos genéticos (vírus e plasmídeos) como arma biológica contra outras bactérias. Assim, estes resultados são de grande importância e precisam de ser futuramente mais explorados.

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CONTENTS

CHAPTER I:

1 INTRODUCTION

1.1 SOCIAL BEHAVIOURS IN BACTERIA

Ever since the first observation of bacteria, by Antonie van Leeuwenhoek in 1676, these microorganisms have been regarded as free-living single-cells, and mostly described as planktonic organisms. In fact, bacteriology has traditionally been focused on a single-cell pure-culture concept based on Koch's postulates (Madigan and Martinko 2006). Only more recently has the social nature of bacteria started to be unveiled. Nowadays, there is a crescent awareness about the communicative and cooperative abilities of bacterial cells to coordinate a multiplicity of multicellular behaviours.

There are several examples of bacterial social behaviours, including, shelter formation, scavenging and foraging, dispersal and communication. Biofilms, for instance, are complex shelter-like structures composed by a single, or even multiple, species of bacteria, surrounded by an extracellular polymer matrix (Costerton, Lewandowski et al. 1995, O'Toole, Kaplan et al. 2000).Bacterial biofilms provide resident bacteria with protection against environmental hazards, like the action of antibiotics, and also assure food accessibility (Crespi 2001). Pathogenic bacteria have also been described to display scavenging behaviours (while infecting an host), for example, in the acquisition of limiting nutrients, such as iron, using siderophores as chelating agents (West, Griffin et al. 2007). Myxobacteria have been described to engage in complex multicellular behaviours such as foraging and dispersal – such bacteria can engage in coordinated attacks that lead to the consumption of prey from different bacterial species such as *Escherichia coli* (Dworkin 1996). In addition, Myxobacteria are able to form fruiting bodies, which require a complex cellular organization and differentiation to form structures responsible for the dispersal of reproduction spores (Shimkets 1990).

Quorum-sensing is a mechanism of bacterial communications that promotes the coordination of multicellular behaviours such as bioluminescence (Ruby 1996) and virulence (Williams, Camara et al. 2000), resulting from the assessment of local bacterial density. In this thesis, other multicellular behaviours will be explored: environmental detoxification, specifically, resulting from the effect of antibiotic resistance factors, and allelopathy (Multicellular behaviours of bacterial populations are reviewed in (Crespi 2001, West, Griffin et al. 2006, West, Diggle et al. 2007, Diggle 2010)).

Bacterial social behaviours have a significant impact on human society, both positively and negatively. An example of bacterial behaviour with a negative impact on human life is the emergence of antibiotic resistance, which is currently a major health threat (reviewed, for example, in (Ventola 2015, Ayukekbong, Ntemgwa et al. 2017, Dodds 2017)) and normally envisioned as an asocial behaviour (Conlin, Chandler et al. 2014). However, several recent studies have shown that it is not so. The vulnerability of bacteria to antibiotics can be adjusted according to their social context. Indeed, several works have demonstrated the ability of antibiotic-sensitive strains to survive in an antibiotic enriched environment, if a resistant strain is present in their vicinity. For example, norfloxacin sensitive cells were able to survive increasing concentrations of that antibiotic, due to the emergence of spontaneous mutants that became resistant to norfloxacin and, paid a significant cost to protect the remaining sensitive cells (Lee, Molla et al. 2010). Also, sensitive cells were able to survive in environments enriched with β-lactam antibiotics due to the action of detoxifying enzymes (β-lactamases) produced by resistant cells. Observations with this class of antibiotic resistance factor include survival of sensitive strains when the resistance factor was overexpressed and encoded on artificial plasmids, whether the sensitive strains was of the same species as the resistant strain (Dugatkin, Perlin et al. 2005, Clark, Alton et al. 2009) or from a different species (Perlin, Clark et al. 2009). Furthermore, sensitive cell-survival to β-lactam antibiotics was also detected on *in vivo* polymicrobial infections (Brook 2004). This extensive and rather frequent protection of sensitive cells from the action of β-lactam antibiotics is alarming since this class of the antibiotics is still one of the first-line choice against bacterial infections (Llarrull, Testero et al. 2010), and it requires a further and deeper understanding of the subjacent ecological and evolutionary driving forces.

Progress has been made towards the understanding of the genetic regulation and mechanisms underlying bacterial physiology, as well as the impact of such beings on a diversity of fields, including environmental issues and health threats. In comparison, relatively little attention has been paid to bacterial social behaviours and the evolutionary forces driving them. Furthermore, social evolution theory was mostly developed by macrobiologists to explain well-known animal behaviours (Crespi 2001, West, Diggle et al. 2007). It is now a challenge to study the large diversity of social

behaviours discovered in bacterial populations and trying to assert whether the theory can generally apply to other taxa. The biology of bacteria offers amazing opportunities, since bacteria are a valuable tool for experimental evolution and behavioural studies, but also new challenges. For example, the ability of bacteria to horizontally transfer genetic material raises new questions and challenges (West, Diggle et al. 2007).

As different authors have already stated: "*this is an exciting time*" both for microbiologists in general, but mostly to researchers interested in the social behaviour of microorganisms (West, Griffin et al. 2006, Diggle 2010). In fact, not only do we have new skills and useful tools to test the existing theory on social behaviours, but we also realize better than ever the importance of understanding and integrating our knowledge on bacterial behaviour.

1.2 Classification of Social Behaviours and Social Evolution Theory

1.2.1 Classification of Social Behaviours

A behaviour can be classified as social if it has an effect on both the actor – individual that performs the behaviour – and the recipient – individual that is affected by such behaviour (Hamilton 1964, Hamilton 1964, Hamilton 1970). The classification of social behaviours is then based on the fitness impact a given behaviour has on both the actor and recipient. The fitness of a given individual can be generally defined as its reproductive success. When a behaviour increases the fitness of the recipient, it can be classified as either mutually beneficial, when it also increases the actor's fitness, or as altruistic, when it results in a decrease of the direct actor's fitness. Conversely, when a behaviour decreases the fitness of the recipient it can be classified as either spiteful, when it also decreases the direct fitness of the actor, or selfish, when it increases the direct fitness of the actor (Hamilton 1964, Hamilton 1964, Hamilton 1970).

The term cooperation is often used to refer to any behaviour that increases the recipient's fitness, and can thus be either mutualistic or altruistic, depending on the impact for the actor (West, Griffin et al. 2007). On the other hand, the term harmful behaviour can be used to refer to any behaviour that decreases the recipient's fitness,

and can thus be either spiteful or selfish, again depending on the impact for the actor (Hamilton 1970).

1.2.2 Social Evolution Theory

The evolution of behaviours with a positive outcome for the actor can be relatively easy to understand. Since natural selection favours the fittest individuals, any behaviour that increases the actor's direct fitness can potentially be favoured. The existence of behaviours with a negative impact on the direct fitness of the actor, in the other hand, is less easy to understand (West, Griffin et al. 2006, Diggle 2010). Therefore, there are two possible explanations for the evolution of social behaviours, each of them based on how social behaviours can affect the fitness of the actor (West, Griffin et al. 2006, West, Griffin et al. 2007).

The first explanation is based on how a behaviour affects the direct fitness of the actor, which can broadly be viewed as the direct effect a behaviour has on the reproductive success of the individual that cooperates (actor). In this case, the behaviour can be maintained if the benefit, of performing the behaviour, outweighs the cost it imposes (West, Griffin et al. 2006, West, Griffin et al. 2007). In this scenario, the reason for the maintenance of the behaviour is apparently straightforward, since it has a positive impact on the fitness of the actor. Thus, the selection of mutually beneficial and selfish behaviours is relatively direct. However, even in this apparently more straightforward situation, the explanation is not completely satisfactory, since a population of individuals that cooperate, for example, can be at risk from being invaded by selfish individuals, which can benefit from the behaviour without paying the associated costs (West, Griffin et al. 2006). I will further develop this matter later.

On the other hand, the existence of costly behaviours, such as altruism and spite, can be more difficult to understand. Selection of altruism amongst living organisms has for long posed a problem to evolutionary biologists. Darwin himself identified altruism as a key problem to his theory and even suggested that certain characteristics could be favoured when they improved the reproductive success of relatives. Darwin, however, could not expand this idea, because gene were still unknown to him. The hypothesis that altruism, when directed towards relatives, can be selected was rigorously formulated by

William Hamilton in 1964, with the inclusive fitness hypothesis, which resulted in the Hamilton's Rule. Such formulation solves Darwin's problem since it shows that genes and not individuals have to be selected: an altruistic behaviour can be selected, even when costly to the actor, if it is advantageous for the altruistic allele. As such, the second explanation for the evolution of social behaviours is based on the impact a given behaviour has on the indirect fitness of the actor. That is, the cooperative behaviour can be maintained, even when directly costly to the actor, if it benefits other individuals in which the probability of carrying the same cooperative gene is higher than for a random individual in the population (r > 0). That is, because it leads to an increase in the reproduction of the actor's relatives resulting in an increase in the representation of its genes in the genetic pool of the population.

In sum, behaviours can be favoured by natural selection when the inclusive fitness of the actor increases, the inclusive fitness being the sum of the direct fitness (impact on the reproductive success of the actor) and indirect fitness (impact on the reproductive success of individuals genetically related to the actor). The key point is that even behaviours detrimental for the actor can be selected if they lead to an increase in the frequency of the actor's gene in the population (through kin individuals). Hamilton (1964) showed that if the force of selection of a given behaviour is weak, that behaviour is favoured if:

$rB - C > 0$, Equation 1

where C is the cost suffered by the actor, B is the benefit gained by the individual the behaviour is directed towards (recipient), and r is the genetic relatedness between the actor and the recipient. Altruistic behaviours can be favoured if the actor increases the fitness of relatives (B > 0 and r > 0 so that rB > C).

According to Hamilton (1970), there is another through which behaviours that decrease the fitness of the actor can be favoured by natural selection. Spiteful behaviours can be favoured if they reduce the fitness of non-related individuals (B < 0 and r < 0 so that again rB > C). Here, relatedness r can be negative if the spiteful

behaviour is directed towards individuals with lower probability of carrying the spiteful gene than a random individual in the population (Hamilton, 1970); this will be further explained at section 2.4.

1.2.3 The Problem of Cooperative Behaviours

The selection of cooperative behaviours, and more so, that of altruism, is thus connected to the way in which such behaviours are going to affect the reproductive success of the actor's relatives. The simplest reason for individuals to be genetically related is to be genealogically related (kin), and that is the reason why the hypothesis is often referred to as "kin selection", a term first coined by John Maynard Smith (Smith 1964). Kin selection can occur via two mechanisms: 1) kin discrimination, when the cooperation is preferentially directed towards kin; or 2) limited dispersal, when relatives remain in close physical proximity and thus obtain a privileged access to the benefits of cooperation. This last mechanism is probably of great importance for bacteria, because bacterial asexual reproduction results in direct descendants of a progenitor bacteria remaining in close physical proximity from one another (when in a structured habitat), hence cooperative behaviours would be directed preferentially towards spatially close kin.

However, individuals in close physical proximity to the cooperating individuals may not always be genetically related. As mentioned previously, a population of cooperators is at risk of being invaded by selfish individuals (cheaters) that benefit from the behaviour but do not cooperate (West, Griffin et al. 2006). In bacterial populations, such individuals can arise through spontaneous mutations that inactivate the gene responsible for the cooperative behaviour, or through migration, from a different bacterial lineage. In this scenario, the cooperative individual pays the cost associated to the behaviour while benefiting individuals that are not kin. In social sciences, this problem is known as the "Public Goods Dilemma", when individuals would benefit from cooperating, but cooperation is not stable, because each individual can gain a transient advantage, by selfishly pursuing their own interests (Dionisio and Gordo 2006, West, Griffin et al. 2007). Therefore, the problem becomes slightly different, although it

persists. Facing the possibility of invasion by cheaters, what evolutionary forces favours the selection of cooperative behaviours?

One possible approach, for the selection of cooperation, would thus be for individuals that perform the cooperative behaviour to preferentially direct it towards non-kin individuals that share the same cooperative gene. This kind of discrimination is in many ways similar to the kin discrimination previously described, but in this case genetic relatives are not necessarily genealogical related (West, Griffin et al. 2007). This form of genetic discrimination could require the same gene to be both responsible for the expression of the cooperative behaviour and, simultaneously, encode a phenotypic trait readily recognizable by individuals with the same gene. Dawkins was the first one to use the term "greenbeard" as an illustrative example of such discriminations (Dawkins 1976).

Alternatively, similar discrimination mechanisms could also be used, even with no directly detectable "greenbeard", that is even in cases where the cooperation gene does not encode simultaneously a phenotypical tag mark. For example, bacteria can have a different genetic background, belonging to different lineages or even different species, and still share common genes, which they acquired through horizontal gene transfer. In this case, the ability of certain bacteria to share the cooperative genes through horizontal gene transfer (such as conjugation) could enable the cooperative population to increase relatedness of close-by individuals (Mc Ginty, Rankin et al. 2011, Mc Ginty, Lehmann et al. 2013). Let's recall that relatedness is a measurement of genetic similarity, and in bacteria it is measured at the locus that determines the trait of interest, in this case, the gene that encodes the cooperative behaviour. Therefore, bacteria that do not carry the cooperative gene are unrelated to the cooperative ones ($r \approx 0$), but those that received the cooperative gene through horizontal gene transfer are as related to the cooperators as direct descendants ($r=1$). In this sense, the horizontal transfer (which is common among bacteria but rare of absent in the rest of living world) of cooperative genes would ensure that local relatedness would increase, hence guarantying that the cooperative behaviour was preferentially directed towards related individuals.

Another possible approach for the selection of cooperation, could also be for cooperators to regain a direct fitness benefit, and would involve mechanisms

responsible for enforcing cooperation and removing the advantage of cheaters, either by rewarding cooperators or punishing cheaters. Smith (2001) hypothesised that the horizontal transfer of cooperative genes between different bacterial lineages could be used as a mechanism to enforce cooperation. According to this hypothesis, the cooperative strain would regain direct fitness benefits by forcing cheaters to become cooperators. This hypothesis was formulated to explain the maintenance of mobile genetic elements on bacterial populations (see section 4.1).

There is yet another phenomenon that can explain the selection of cooperative behaviours, even without preferentially directing the public goods toward relatives and without a mechanism of enforcement. In nature, a population of bacteria is likely to be subdivided into different subpopulations spread across different ecological patches, and composed of different frequencies of producers and cheaters cells. In each subpopulation, cheater cells may have a selective advantage, depending on the demographic conditions, because they do not pay the cost of cooperation. Despite that, the overall population of public good producers is bound to increase. This phenomenon is called Simpson's Paradox, and results from the fact that subpopulations with higher levels of cooperation have more descendants than those where cheaters are more represented. This paradoxical phenomenon is called Simpson's Paradox after the British statistician Edward H. Simpson , who first described it in the early 1950's (Simpson 1951). The phenomenon was later demonstrated in the context of ecology, by D. S. Wilson (Wilson 1975). Later is has been shown that this ecology effect is in fact another form of kin selection; the reason is that the total frequency of altruists increases only if the variance of the frequency of altruists between groups is (much) higher than the variance within groups, a mathematical way of saying that relatedness is high (Frank 1998). Experimental results with synthetic microbial systems using *Escherichia coli* artificial strains have already demonstrated the occurrence of such phenomenon within bacterial populations (Chuang, Rivoire et al. 2009).

Despite of the hindrances inherent to cooperation, and even more so to altruism, such behaviours have been frequently observed, studied and described, both in macroscopic and microscopic organisms. Pathogenic bacteria often cooperate, in several different ways, to efficiently invade and infect its hosts. *Pseudomonas*

aeruginosa, for instance, can produce siderophore molecules that are secreted to the environment. Such molecules are metabolically costly, and once in the environment, act as chelating agent that binds iron molecules, otherwise insoluble or host-bound, so that the iron can then be taken up and used by all the bacteria in the vicinity. Iron is a major limiting factor for bacterial growth, and siderophore production is a scavenging mechanism that benefits the whole local population, hence being considered cooperative (Ross-Gillespie, Gardner et al. 2007, West, Griffin et al. 2007, Ross-Gillespie, Gardner et al. 2009).

1.2.4 The Problem of Spiteful Behaviours

Spite, on the other hand, is less readily observed in nature, and its existence has been the centre of debates, mainly due to a general lack of theoretical consensus and of empirical evidences on the subject (Lehmann, Bargum et al. 2006). In fact, after his initial formulations Hamilton himself anticipated that spiteful behaviours could not be selected in nature (Hamilton 1964). Since spite is defined as a behaviour with a negative impact on both the actor and the recipient individuals, the lack of selective forces enough to favour such behaviour seems natural. In other words, if a behaviour has a negative benefit ($B < 0$) for the recipient, and a positive cost ($C > 0$) for the actor, what forces could possibly ensure its selection? Hamilton latter argued that spite should in fact be observed in nature, when preferentially directed toward non-related individuals (Hamilton 1970). As such, the forces favouring the selection of spite would be the opposite of those ensuring the maintenance of altruistic behaviours (Lehmann, Bargum et al. 2006). As previously mentioned, altruism is favoured when the behaviour is directed toward kin ($r > 0$). Spiteful behaviours, on the other hand, are favoured when directed towards non-kin, a case in which relatedness is negative ($r < 0$).

Relatedness is measured population-wide as a regression coefficient, and may, as such, assume negative as well as positive values. There is negative relatedness when a target individual in the population is less likely to share the same genes with the actor than the average individuals in the population (Foster, Wenseleers et al. 2001, Gardner and West 2004). Spite is then favoured by natural selection, when directed towards non-

relatives ($r < 0$), because it leads to a decrease in the frequency of competing alleles in the genetic pool of the population (Gardner and West 2004).

One of the key issues when it comes to classify a behaviour as spiteful is that it is difficult to distinguish between true spite, which has no benefit for the actor, and cases of mere selfishness, in which the actor can be initially harmed by the behaviour but ends up benefiting, in the long term, from it. Several behaviours have been, therefore, considered spiteful by some authors and merely selfish by others, because despite imposing a significant cost on the recipient the actor is more likely to benefit from the behaviour (Foster, Wenseleers et al. 2001). Hamilton considered that a behaviour was spiteful when it resulted in a decrease of the direct fitness of both the actor and the recipient (individual reproduction), while other authors (Pierotti 1980, Waltz 1981, Trivers 1985) considered a spiteful behaviour to result in the decrease of the inclusive fitness of both actor and recipient (reproduction of all individuals that share the actor's genes). While Hamilton's spite can be potentially favoured by natural selection, because the genes of the actor prevail in the population, spite according to the second definition, is doomed to be negatively selected, because neither the actor nor its relatives are favoured by the spiteful behaviour (Foster, Wenseleers et al. 2001).

It has been proposed that hosts may use parasites as biological weapons, to decrease the fitness of competitors (Rozsa 2000, Dionisio 2007). Bacteria, for example, could use bacteriophages (phage for short) to lower the fitness of susceptible competitors. Bacteriophages are viruses that infect bacteria. The λ phage, has been used as a proof of concept (Dionisio 2007). This virus is a temperate phage, which means that, instead of merely entering the bacterial cell, producing viral progeny and releasing it through the lysis of the host cell (lytic cycle), the λ phage can, upon the entrance into the host bacteria, act as a temperate phage and remain dormant in a prophage state. In this dormant state, the λ phage can be vertically transmitted to daughter cells during the replication process, additionally assuring that descendent cells have immunity against superinfection (Oppenheim, Kobiler et al. 2005). In other words, as long as λ phage resides within a lysogenic bacterium, it prevents similar λ phages from initiating a lytic cycle, and killing its host. One of the reasons pointed by Hamilton's for the rarity of spiteful behaviours is the lack of efficient mechanisms for an actor to recognize if

another individual is related to itself. With parasites such as the λ phage, such a problem is solved: this virus is only capable of entering λ-free bacterial cells, hence bacteria unrelated to cells carrying the virus in the chromosome. If the lytic cycle of the phage is resumed within the lysogenic bacterium, the actor cell will eventually suffer lysis and releases phage progeny into the environment. Those free phages will then be able to act as allelopathic agents, infecting and harming non-relatives of the actor, since its own relatives will carry a prophage and hence be protected against superinfection (Dionisio 2007]). This example illustrates the statement that "altruism and spite represent two sides of the same coin" (Lehmann, Bargum et al. 2006).

When a lysogenic bacterium lyses and releases phages into the surrounding environment it pays a very high cost (death) to harm susceptible bacteria in its vicinity, and as such, the behaviour can be deemed as spiteful. From the actor relative's point of view, however, the lysogenic bacterium suffers a cost to improve their fitness, and as such the behaviour is altruistic. The same behaviour can thus be seen as both cooperative and harmful, since the behaviour is directed towards negatively related individuals – ones that do not carry the phage – which ultimately leads to an increase in the fitness of the positively related individuals – those that carry the prophage – in the population (Lehmann, Bargum et al. 2006). This scenario demonstrates that bacterial parasites, such as bacteriophages, can potentially be maintained in bacterial population as helpful instruments in bacterial social struggles.

1.3 Maintenance of Mobile Genetic Elements in Bacterial Populations

In addition to their chromosome(s), bacteria often harbour extra-chromosomal genetic elements, such as plasmids, temperate bacteriophages and transposable elements. Such elements carry genes that are by definition non-essential to the bacterial host and that are accessory to their chromosome. Moreover, mobile genetic elements (MGEs) are the main agents responsible for the ability of bacterial cells to share genes between different lineages. Hence, bacteria are organisms able to transmit genes both vertically, from parental to descendent cells, and also horizontally to cells of different

lineages. This horizontal transfer of genes can be accomplished through three different mechanisms, depending mostly on the nature of the foreign genetic material. Naked DNA can be taken up from the environment of a bacterial cell by a mechanism called transformation. The transfer of genes through a bacteriophage vector is referred to as transduction, while the transfer mediated by plasmids is called conjugation (Juhas 2015). Horizontal gene transfer and consequent uptake of a MGE can be either beneficial or detrimental to a bacterial host.

MGEs do not necessarily share the same interests with the host core genome and are mostly infectious agents. Besides, different MGEs impose different costs on the host. Bacteriophages can be either virulent or temperate. A virulent phage "infectious cycle" begins with the attachment to the host bacteria and subsequent penetration of its genetic material into the host cell. Following the invasion of the bacterial cell, the phage then replicates inside its host producing viral progeny that will, ultimately, be released to the environment, resulting in the death of the host bacteria by lysis.

A temperate phage, on the other hand, has two possible "infectious cycles". The lytic pathway is similar to the infectious cycle of the virulent phage: after the production of viral progeny inside the bacterial cell, the progeny is eventually released through the lysis of the cell host. In the lysogenic pathway, the genetic material introduced inside the bacterial host is stably incorporated as a prophage – either integrated in the bacterial chromosome or as an episome. In this latter scenario, it is said that the bacterium becomes lysogenic. In this state, the phage genome is allowed to replicate alongside the host genome and can be transmitted vertically, to descendent cells. While in the prophage state, beneficial genes carried by the phage can potentially be useful to the host, and can include genes for bacterial virulence and antibiotic resistance. Later, if the prophage remains functional, it can be triggered at any time, and resume the lytic pathway, ultimately resulting in the death of the host cell (Oppenheim, Kobiler et al. 2005). The death of the bacterial cell then results in the release of the viral progeny, and restarts the horizontal transfer of genes. Therefore, despite being potentially useful to a host bacterium, phages impose a life-or-death dilemma, and the horizontal gene transfer resulting from transduction always impose a very high cost on the host, because it results in the death of that host.

Other MGEs, such as conjugative plasmids, do not impose such a life-or-death dilemma, but are still a potential burden to their host cells. When inside a bacterium a plasmid will use the host's machinery to replicate and be vertically transmitted to daughter cells. Additionally, conjugative plasmids will use the host's resources to build the physical structures required for their horizontal transfer (sex pili). Thus, replication, maintenance and transmission of conjugative plasmids will always imply a metabolic cost for the bacterial host, which may decrease the host's fitness. However, this cost can be low or easily eliminated after some generations of co-evolution between a plasmid and its bacterial host (Bouma and Lenski 1988, Modi, Wilke et al. 1991, Dahlberg and Chao 2003, Dionisio, Conceicao et al. 2005)

Aside from their potential costs, MGEs can also carry genes that encode useful traits for the bacterial host in specific circumstances, and as a result MGEs have been referred to as "agents of open source evolution" (Frost, Leplae et al. 2005). Such traits may include antibiotic resistance, pathogen virulence, allelopathy or secondary metabolism functions. Thus, despite the non-essential nature of MGEs, they can be sometimes regarded as mutualistic agents. In addition to the benefits for the bacterial host, these traits may also influence the fitness of other bacteria in the vicinity, either positively or negatively. Furthermore, it has been argued that MGEs promote social traits because of the local effect they have on the genetic structure of bacterial population (Rankin, Rocha et al. 2011).

1.4 Mobile Genetic Elements as drivers of Bacterial Sociality

1.4.1 Cooperation

One of the main mechanisms of bacterial cooperation is the production of "public goods" – molecules whose effect is readily available for all the cells in the vicinity. Public goods molecules can have different functions, such as virulence or antibiotic resistance, many of which will increase bacterial fitness, not only that of the producer but also that of neighbouring individuals.

Public goods may be costly to the bacterial producer. Therefore, and similarly to other forms of cooperation, production of public goods is at risk from being exploited by selfish individuals (cheaters), which are able to benefit from the effects of the public good without paying the cost associated with its production. Cheaters will have a fitness advantage relatively to that of the producers, and thus will potentially be able to win when competing with cooperative bacteria.

Genes encoding public goods are often carried on MGEs (Rankin, Rocha et al. 2011). In fact, it has been observed that different functions are encoded on the vertically transmissible core genome, which mostly encodes essential cellular processes, while the horizontal transmissible accessory genomes (which includes MGEs) encode a variety of recently acquired functions, with an overrepresentation of secreted products (Nogueira, Rankin et al. 2009). The diversification of *E. coli* pathotypes is an alarming example of the acquisition of new genes with extracellular effects, through horizontal gene transfer (Kaper, Nataro et al. 2004). Moreover, it has been proposed that the maintenance of MGEs may be associated with the enforcement of cooperative behaviours, in bacterial populations (Smith 2001).

In his study, Smith (2001) hypothesized that if genes encoding a public good are carried by a plasmid, it has the potential of being horizontally transferred to cheaters. By doing so, the cooperative individuals would use their MGEs as a mechanism to enforce the cooperative behaviour on the local population. However, this hypothesis presents some limitations. For instance, it has been demonstrated that if the population was invaded by another plasmid that does not carry the cooperative gene the bacterial population would still be at risk from being outcompeted by cheaters, only this time the social dilemma would repeat itself at the level of the plasmid (Mc Ginty, Rankin et al. 2011). In other words, the enforcement of cooperation through horizontal gene transfer, via plasmids, when cheaters do not carry plasmids is possible but short-lived, since the cooperative population remains at risk of being invaded and outcompeted by cheater cells that carry non-cooperative plasmids. Then, why are public good genes so over represented on MGEs? Why are genes not sequestered into the bacterial chromosome?

Two complementary mechanisms through which public good genes can be maintained, via plasmids, have been proposed (Mc Ginty, Lehmann et al. 2013). First, through infectious transfer, meaning the spread of plasmids into previously uninfected cells directly increases the number of plasmid carriers. Second, through kin selection, since plasmids can increase their local relatedness, ensuring the beneficial effect of public goods is helping related individuals. Genetic relatedness describes the statistical association between genes in different individuals (Hamilton 1970), and is always measured at the locus of interest. When the gene for a public good is carried by a plasmid, the locus of interest is on the plasmid. Since the plasmid can be horizontally transferred, bacteria from different lineages (initially, r=0) can become related (r=1) after receiving the plasmid. It has been demonstrated that horizontal transfer can increase the whole-group relatedness at the plasmid level (Mc Ginty, Lehmann et al. 2013), which changes the population assortment and can ensure that the cooperative behaviour is favoured via kin selection. Experimental results, have indeed demonstrated that infectious transfer can increase cooperation. In unstructured populations, this effect is short-lived, because infectious transfer can benefit any plasmid. This means, that a plasmid which carries a costly public-good gene can be outcompeted by a plasmid that does not, since the latter is able to be transferred, but does not bear the cost of public-good production (Mc Ginty, Rankin et al. 2011, Dimitriu, Lotton et al. 2014). However, in structured populations, horizontal transfer favours public good production by increasing the feedback of public goods benefits preferentially to producer alleles (Dimitriu, Lotton et al. 2014).

In sum, the association between the public good overrepresentation on MGEs and its impact on bacterial social behaviours operates at different levels. MGEs not only offer the possibility to increase the number of cooperative individuals on a bacterial population (via infectious transfer), thus forcing previously cheater cells to become cooperators; but also guarantees that the benefits of public good production is directed to genetically related individuals, by increasing the local relatedness of a population.

1.4.2 Harmful Behaviours

In the previous section we analysed situations in which a population, despite being adapted to its environment, is invaded by less adapted individuals. In this scenario, the pre-existing population is considered to be well adapted to its environment because it produces a public good that modifies the surrounding environment to its own profit (for example an antibiotic resistance factor which detoxifies the environment or a virulence factor that allows the infection of a host). The less adapted individual invades the pre-existing population because it can benefit from the use of the public good, without paying the respective cost, hence having a higher relative fitness.

Alternatively, less adapted individuals can invade a pre-existing population through the modification of the environment, transforming it into a more benefiting configuration to themselves. There are two ways of accomplishing this: i) Niche construction, for example when organisms build nests, spider webs or beaver dams; or ii) Niche deterioration, for example, when bacteria produce toxins (Brown, Fredrik Inglis et al. 2009). Niche deterioration can also be referred to as interference competition or allelopathy (Fitter 2003).

One possible form of chemical allelopathy is through the production of bacteriocins, or other toxins, to kill individuals in the surrounding population. Bacteriocins are small peptides or heat stable proteins commonly coded by bacterial strains that kill other bacterial cells, usually from the same species (Riley and Wertz 2002, Gardner, West et al. 2004). The release of bacteriocins comes to a cost for bacterial cells, since it may imply the death of the carrying host, yet it is beneficial for bacterial cells of the same lineage, which carry specific antidote coding genes. Colicins are a specific class of bacteriocins which are produced and act against *Eschecichia* coli and other related *Enterobacteriaceae.* At a given time, only a small proportion of the whole colicinogenic population produces and releases the bacteriocin. Other colicinogenic bacteria present in the population are immune to its effects, while sensitive bacteria are killed by its action. The death of sensitive cells may help immune cells, for example because more resources are left. This helpful action towards relatives led earlier authors to describe this behaviour as altruistic (Smith 1978). Yet, the release

of such molecules invariably results in the death of the producing cell, which means the colicinogenic bacteria that displays the behaviour pays a very high cost. As such this behaviour is clearly spiteful, because it is costly for the producer and harms other members of the population (although it can be seen as altruistic, from the point of view of immune cells from the same bacterial lineage of the producer cell, because they benefit at its expense).

Colicin production and immunity are mostly carried by plasmids (Chao and Levin 1981). Since plasmids can carry both the bacteriocin and the corresponding antidote gene (Riley and Wertz 2002), if a cell coding for the bacteriocin releases the toxin, it kills members of the population that do not bear the plasmid. This favours the individuals that carry the plasmid. As such, we can also see this behaviour as being a "greenbeard", because producers of the bacteriocin preferentially benefit individuals carrying the same complex of genes (toxin-antitoxin) (Gardner and West 2010).

Bacterial spiteful extracellular products are frequently associated with MGEs (Rankin, Rocha et al. 2011). MGEs can be used by bacterial hosts as harming agents, whether directly, as when releasing phages, that will act as biological weapons against neighbouring bacteria (mentioned in section 2.4), or indirectly, as for the bacteriocins carried by plasmids.

1.5 The effect of Demographic factors on bacterial Social Behaviours

The impact that social behaviours have on the individuals of a bacterial population can be affected by demographic factors, such as the genetic composition of the population, the frequency or ratio among genetically unrelated organisms, as well as the population's density and structure. The effect of demographic factors on the selection of social behaviour has been the focus of theoretical and experimental attention (Dugatkin, Perlin et al. 2005, Ross-Gillespie, Gardner et al. 2007, Ross-Gillespie, Gardner et al. 2009). In fact, several works have demonstrated the influence of habitat structure, population density and frequency on the selection of bacterial social behaviours (Chao and Levin 1981, Dugatkin, Perlin et al. 2005, Ross-Gillespie, Gardner et al. 2007, Ross-

Gillespie, Gardner et al. 2009). Bacteria offer good opportunities for the experimental study of demographic factors, mainly due to their large population size and rapid generation time.

Habitat structure may be defined as the patterns of genetic variability within and between groups (Frank 2010). In unstructured habitats, different individuals can randomly interact with one another and competition is global, while in structured habitats one individual can only interact with other individuals in the vicinity and, therefore, competition is local. In the specific case of bacterial cell culture, a liquid culture with constant agitation is considered to have no structure, while the growth of bacterial population over a solid medium is an example of a structured habitat. This difference in the level of structure is likely to have an impact on the outcome of competitions between cooperators and cheaters, as explained bellow.

If a public good diffuses freely across the environment (unstructured habitats) it will result in a widespread and diluted effect of cooperation. The benefits of such behaviour will be randomly distributed among cooperative and cheater individuals alike. In structured habitats, the public good will be concentrated around producer colonies, therefore, cheaters located in the vicinity of producers will benefit more from this structured allocation. This general prediction can be affected by the nature of the public good. For instance, if the public good is partially sequestered by producers so that they gain a privileged access to its effects, benefits for cheaters can be more difficult to obtain. For example, β-lactamase molecules produced by Gram negative bacteria are primarily sequestered within their periplasm (space between the inner and outer membrane) and, although some of those molecules can diffuse and detoxify the surrounding environment, the producer cells have a privileged access to its effect. Another possible peculiarity would come from a strong effect of the public good. Bacteria can secrete molecules able to detoxify their surrounding environment, β-lactamase, for example, can clear the environment from the effect of β-lactam antibiotics. If such detoxifying molecules have a weak power of detoxification, unstructured environments will be less favourable to cheaters than structured ones. However, if the detoxification power is strong, global detoxification in an unstructured habitat may be more efficient.

Maintenance of cooperation through horizontal gene transfer is also affected by the habitat structure. In unstructured habitat, the infectious transmission of a conjugative plasmid, encoding a public good, will ensure a transient enforcement of the cooperative behaviour, by forcing previously non-producer cells to become producers. However, in structured habitats, the horizontal transmission of the public good gene will change the local population assortment, turning cells that were initially non-relatives into relatives (which carry the public good gene). Thus, the individuals in the vicinity of cooperators will become genetically related, which guarantees that cooperation is preferentially directed towards relatives (Dimitriu, Lotton et al. 2014).

The effect of habitat structure in social behaviours is not restricted to cooperative behaviour. In a structured habitat, when a bacterial cell is killed through a harmful behaviour, such as the production of bacteriocins or phages, it leaves behind unused resources on the proximity of cells displaying the harmful behaviour. In unstructured habitat, freed resources are randomly distributed and equally available to all individuals in the populations. (Chao and Levin 1981) showed that while in unstructured habitat, colicinogenic bacteria only had an advantage when initially common, in structured habitat they had an overall advantage (higher when rare). These results also show that different demographic factors can interact between themselves, influencing the outcome of a social interaction. Another example, was provided using a community constituted of three types of bacterial strains: colicinogenic, colicin-sensitive and colicin-resistant. This community reflects a classic example of the rock-paper-scissors relationship. In theory, the resistant strain is able to outcompete the colicinogenic strain, because it does not carry the costly Col plasmid (responsible for the production of the colicin). The colicinogenic strain outcompetes the sensitive strain, because colicins lead to sensitive's death. And, sensitive strains can outcompete the resistant strain, because the resistance mutation affects crucial physiological traits of the bacteria and is thus costly. In structured habitat, where competition is local, this balance is observed resulting in the maintenance of diversity. In unstructured habitat, however, the well-mixed system results in a loss of diversity, since colicinogenic bacteria rapidly drive the sensitive strain extinct, and are then outcompeted by the resistant strain (Kerr, Riley et al. 2002).

Population density also affects bacterial social interactions. In fact, when a population of public good producers is being exploited by cheaters, the latter strain gains a greater advantage when cell density is higher. For example, cheaters exploitation of *Pseudomonas aeruginosa* siderophores has a positive density dependence (Ross-Gillespie, Gardner et al. 2009). This increased advantage at higher population densities can be due to several factors, for instance, because higher cell densities may translate into production of more public goods. Also, higher cell densities will increase the number of cells each cheater can interact with, increasing the odds of approaching a producer cell. Such positive density dependence, however, is predicted to be weaker in more structured habitats where cheaters only can gain an advantage when in close physical proximity to producer cells (Ross-Gillespie, Gardner et al. 2009). This means that, in structured habitat, high densities can be more advantageous for cooperation, since producer cells will be more likely to have a privileged access to the public goods; moreover, because there is limited dispersal, producer cells are more prone to be cooperating with relatives.

Density is a clinically relevant factor when it comes to bacterial infections. For example, intra-abdominal infections, such as appendicitis and peritonitis, can result in a high density of bacteria in the infected area. Such high local burden has been medically associated with a higher tolerance of sensitive bacteria to β-lactam antibiotics (Stevens, Yan et al. 1993). This phenomenon has been designated as inoculum effect and has been demonstrated experimentally. Bhagunde, Chang et al (2010) demonstrated that the killing function of an antibiotic is inversely dependent on the initial bacterial burden. This positive-density dependence of sensitive cell tolerance to antibiotics can be explained by a reduced effective drug exposure, but also due to potential different physiological states of the bacteria and even to biofilm formation (Bhagunde, Chang et al. 2010). It has been shown that this inoculum effect may be observed with all β-lactam antibiotics (Tam, Ledesma et al. 2009). Additionally, the effect was observed with sensitive *Staphylococcus aureus*, which was better able to survive to six different antibiotics when at higher cell densities (Udekwu, Parrish et al. 2009). This survival was mainly attributed to a reduction in the effective concentration of antibiotic in the environment, hypothesized to be due to denaturing enzymes, or the binding of

antibiotic molecules to cellular structures of both viable and dead bacteria (or even cell debris).

The influence of genotype's frequency on social interactions is less straightforward to predict. For instance, the initial frequency of cheaters in a population should not influence the fitness of the strains if selection is weak (Hamilton 1964). However, it has been theoretically predicted that the fitness of cheaters can have a negative-frequency-dependence (Dugatkin, Perlin et al. 2005, Ross-Gillespie, Gardner et al. 2007). This prediction has been experimentally confirmed, by several authors. For example, cheater strains exploiting the detoxification of antibiotic enriched environment, by β-lactamase-producing strains of *E. coli* have a greater fitness advantage when initially rare (Dugatkin, Perlin et al. 2005). Also, the success of cheater exploitation of siderophores produced by *P. aeruginosa* is negatively correlated with initial cell frequency (Ross-Gillespie, Gardner et al. 2007). Nevertheless, the theoretical prediction is that frequency-dependence is conditioned by at least two other demographic factors: 1) Habitat Structure; and 2) Cell Density. In fact, experimental observations have revealed that negative-frequency dependence is stronger in structured than in unstructured habitat. The reason for this is that, in structured habitat, cells in the vicinity of public good producers have a privileged access to it. Also, the strength of negative-frequency dependence is lowered when less growth is allowed. This means that, with lower initial cell densities, frequency dependence should be more evident because more rounds of cellular multiplication and of public good production should have a greater benefit for cheaters. Likewise, in cases in which higher frequency of cooperators lead to a greater population growth, the fitness of cheater cells should be under negative frequency selection, because the higher initial frequency of producer cells the more benefits should the cheaters acquire (Ross-Gillespie, Gardner et al. 2007).

With harmful behaviours, frequency-dependence is also entwined with other demographic factors. For instance, the fitness of colicinogenic bacteria has a negative-frequency dependence only on unstructured habitats (Chao and Levin 1981). In this case, there is a frequency threshold above which the advantage of colicinogenic strain decreases. Contrariwise, due to the amplification ability of phages, lysogenic bacteria

carrying the phage ϕ80 can invade well-mixed (unstructured) populations, when initially rare (Brown, Le Chat et al. 2006).

In sum, bacterial social behaviours can, not only be affected by different demographic factors, but also such factors are bound to have a combined and interactive effect on the fitness of bacterial lineages during the course of social interactions.

1.6 OBJECTIVES

This work focused primarily on bacterial social interactions, especially those driven by mobile genetic elements, such as temperate bacteriophages and conjugative plasmids, to study both harmful and cooperative behaviours in bacterial populations.

Chapter II – Can bacteria use bacteriophages as allelopathic agents?

Chapter III – Can bacteria exploit the cooperative detoxification of the environment? To what extent and how do demographic factor affect such exploitation?

Chapter IV – Can bacteria use plasmids to enforce bacterial cooperation?

Chapter V – Can sensitive bacteria help protect other sensitive bacteria from the effect of an antibiotic?

Across the chapters, special attention will be payed to the effect of demographic factors, such as initial cell density and frequency, and habitat structure (structured versus unstructured, and physically mixed versus physically separated).

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CHAPTER II:

2 TEMPERATE BACTERIAL VIRUSES AS DOUBLE-EDGED SWORDS IN BACTERIAL WARFARE

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João Alves Gama, Ana Maria Reis, **Iolanda Domingues**, Helena Mendes-Soares, Ana Margarida Matos, and Francisco Dionísio

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2.1 ABSTRACT

It has been argued that bacterial cells may use their temperate viruses as biological weapons. For instance, a few bacterial cells among a population of lysogenic cells could release the virus and kill susceptible non-lysogenic competitors, while their clone mates would be immune. Because viruses replicate inside their victims upon infection, this process would amplify their number in the arena. Sometimes, however, temperate viruses spare recipient cells from death by establishing themselves in a dormant state inside cells. This phenomenon is called lysogenization and, for some viruses such as the λ virus, the probability of lysogenization increases with the multiplicity of infection. Therefore, the amplification of viruses leads to conflicting predictions about the efficacy of temperate viruses as biological weapons: amplification can increase the relative advantage of clone mates of lysogens but also the likelihood of saving susceptible cells from death, because the probability of lysogenization is higher. To test the usefulness of viruses as biological weapons, we performed competition experiments between lysogenic *Escherichia coli* cells carrying the λ virus and susceptible λ-free *E. coli* cells, either in a structured or unstructured habitat. In structured and sometimes in unstructured habitats, the λ virus qualitatively behaved as a "replicating toxin". However, such toxic effect of λ viruses ceased after a few days of competition. This was due to the fact that many of initially susceptible cells became lysogenic. Massive lysogenization of susceptible cells occurred precisely under the conditions where the amplification of the virus was substantial. From then on, these cells and their descendants became immune to the λ virus. In conclusion, if at short term bacterial cells may use temperate viruses as biological weapons, after a few days only the classical view of temperate bacterial viruses as parasitic agents prevails.

2.2 INTRODUCTION

Frederick W. Twort, in 1915, and Felix d'Hérelle, in 1917, discovered "a microbe that was "antagonistic" to bacteria and that resulted in their lysis" (Twort 1915, d'Herelle 1917, Duckworth 1976). This kind of microorganism was later termed bacteriophage, which literally means bacteria eater. In 1940, with the advent of the electron microscope, the nature of this microorganism was revealed: it was a virus (Summers 2006).

Bacteriophages (phages for short) are viruses that infect bacteria and can be generally classified as virulent or temperate (for a review see (Campbell 1996)). When a virulent phage infects a bacterial cell, it undergoes replication followed by the release of viral progeny, which results in host death. However, if the phage is temperate, two outcomes are possible after infection of a bacterium. Either there is production of viral progeny and host cell lysis similarly to what occurs for virulent viruses, or lysogeny occurs, meaning that the phage genome is stably incorporated as a prophage in the host cell (either integrated in the chromosome or as an episome). In the latter case, it is said that the host becomes lysogenic. This allows the phage genome to be replicated along with the host genome and consequently transmitted vertically to daughter cells. Some stressful conditions (such as UV radiation or thymine starvation) may induce the production of the viral progeny and, as a consequence, the host cell lysis (Oppenheim, Kobiler et al. 2005).

Temperate bacteriophages can have several ecological roles. The conventional view is that they are either (i) parasites, because they exploit their host for reproduction, or (ii) predators, because bacteriophage replication and release usually kills the host. Lately, it has become evident that bacteriophages and bacterial cells may also establish a mutualistic relationship given that many phages code for virulence factors that will allow the bacteria to successfully infect hosts and expand their distribution (Brussow, Canchaya et al. 2004, Casas and Maloy 2011). Another benefit of harbouring prophages, observed with *Escherichia coli* and several phages (λ, P1, P2 and Mu), is the fact that, during aerobic growth under conditions of continuous carbon source limitation, lysogenic strains have higher metabolic rate than non-lysogenic. Therefore, such lysogens reproduce faster than non-lysogenic strains (Edlin, Lin et al. 1975, Edlin, Lin et

al. 1977, Lin, Bitner et al. 1977), outcompeting them in environments where nutrients are scarce.

More recently, it has been proposed that pathogens and parasites in general may be useful to their hosts as biological weapons (Rozsa 1999, Rozsa 2000, Dionisio 2007). In particular bacteriophages may be useful to bacteria as antagonistic allelopathic entities (Brown, Le Chat et al. 2006). However, this allelopathic agent is not a biochemical agent, but a "replicating agent" antagonistically affecting the growth, reproduction, or survival of other organisms (for reviews with a generalized view for the role of bacteriophages and other agents, see (Brown, Inglis et al. 2009, Brown, West et al. 2009)). In other words, lysogenic cells may use viral particles in a way similar to the use of toxins to kill non-lysogenic susceptible strains, thus possessing a fitness advantage over them. Moreover, the viruses may replicate inside bacteria, which die in process of virus release. Therefore, in contrast to typical toxins, the ability of phages to replicate inside their victims would give an additional advantage to the lysogenic bacterial population (Brown, Le Chat et al. 2006, Joo, Gunny et al. 2006, Dionisio 2007). The amplification of phages inside their victims is particularly significant when only a small number of carriers of bacteriophages invade a susceptible bacterial population: within a few hours, the victims can substantially amplify the number of viral particles (Brown, Le Chat et al. 2006). Alternatively, as mentioned before, temperate bacteriophages can establish a symbiotic relationship with the host. In this case, the cell is not killed and becomes immune to similar viruses.

Bacteriocins are proteins commonly coded in bacterial strains that kill other bacterial cells. In a similar way to what happens with bacteriophages, the antagonistic activity of bacteriocins is usually restricted to members of the same species; therefore, bacteriocins play an important role in competition between conspecifics (Riley and Wertz 2002, Riley, Goldstone et al. 2003). In this paper we sometimes give examples concerning colicins, which, by definition, are bacteriocins produced by *E. coli* (for a review on bacteriocins, see (Baba and Schneewind 1998), and for a review on colicins, see (Cascales, Buchanan et al. 2007)).

The colicin operon includes genes that code for the toxin itself and for immunity to this toxin. The operon of some colicin types also codes for a lysin. This lysin allows the producer to release the toxin to the environment, despite causing its own death.

Therefore, and for similar reasons, the release of these colicins or of bacteriophages is costly to the cell that produces the toxin or the virus, respectively. However, clone mates of the producer are protected from its killing effect because they have the immunity gene. In other words, the antagonistic effect of bacteriocins (or of bacteriophages) is only directed to genetically distinct individuals (Chao and Levin 1981, Kerr, Riley et al. 2002, West, Diggle et al. 2007).

Antagonistic interactions mediated by colicins can be viewed as spiteful interactions (Hamilton 1970, Grafen 1985, Foster, Ratnieks et al. 2000, Gardner and West 2004, Gardner, West et al. 2004). Given that bacteriocins kill susceptible bacteria whereas clone mates of producers are immune to it, the bacteriocin producer and its victims are negatively related (Grafen 1985, Foster, Ratnieks et al. 2000). Gardner and West (2004) noted that there are two main elements involved in bacteriocin-mediated competition ((Queller 1994); see also ref. (West, Pen et al. 2002)): (i) the spatial scale at which competition for resources takes place; and (ii) the fraction of social partners that are clonal kin (Gardner and West 2004, Gardner, West et al. 2004).

In a structured habitat, competition is local. However, in unstructured habitats, all (or most) individuals in a population are social partners and competition is (almost) entirely global. Therefore, structured habitats offer better conditions for the evolution of spiteful behaviour (Hamilton 1970, Grafen 1985, Foster, Ratnieks et al. 2000, Gardner and West 2004, Gardner, West et al. 2004).

There is a second reason why the effect of an antagonistic interaction like the one mediated by bacteriocin (Chao and Levin 1981, Iwasa, Nakamaru et al. 1998, Kerr, Riley et al. 2002) or antibiotic (Wiener 2000) production depends on the structure of the habitat. In these habitats the killing of susceptible bacteria frees unused resources on the proximity of the cells coding for the toxin (Chao and Levin 1981). This is different from what happens in mass habitats, where freed resources are randomly distributed and equally available to all individuals in the population (Chao and Levin 1981).

Both factors (spiteful interaction followed by competition for resources) can lead to the following effects of bacteriocins. In unstructured habitats, (e.g., liquid habitats where individuals affect equally the environment of every other individual), there is advantage of strains coding for bacteriocins if their frequency is above a certain threshold, otherwise bacteriocin production is disadvantageous. This was observed with

the colicin E3 (Chao and Levin 1981). However, in structured habitats (where an individual has a stronger effect on neighbours) bacterial cells coding for colicin E3 are at an advantage even if initially rare (Chao and Levin 1981).

As we have seen, temperate bacteriophages and bacteriocins share several properties. However, they are different in two fundamental aspects. On one hand, bacteriophages may replicate inside victims, and this may lead to an overall increase in the number of bacteriophages; this property confers an advantage to lysogens. On the other hand, temperate bacteriophages, once inside their new hosts, may establish themselves and confer immunity to similar bacteriophages; this property confers a disadvantage to the original lysogenic cells. Actually, genes coding for bacteriocins and immunity may also be transferred between bacterial cells (Brown, Le Chat et al. 2006), but one may expect plasmid transfer rate to be much lower (Gordon 1992, Dionisio, Matic et al. 2002) than in the case of phages.

We thus ask: given that these two properties of temperate viruses originate opposing selective forces, which one prevails? And what is the role of habitat structure? To answer these questions, we study the effect of bacteriophages in structured and unstructured habitats and check the relative importance of virus amplification and lysogenization. We further compare our results concerning bacteriophages with previous results on the effect of bacteriocins (Chao and Levin 1981, Frank 1994, Iwasa, Nakamaru et al. 1998, Kerr, Riley et al. 2002) and other toxins (Wiener 2000).

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Strains

In this study, we used *E. coli* MG1655 Δ*ara* λ^+ streptomycin-resistant (lysogenic strain) (Lys Str^R), *E. coli* MG1655 Δ*ara* rifampicin-resistant (susceptible to λ phage, phage-free strain) (Sus Rif^R), *E. coli* MG1655 Δ*ara* λ^r rifampicin-resistant (phage-free but resistant to λ) (Res Rif^R), *E. coli* MG1655 Δ*ara* λ^+ rifampicin-resistant (lysogenic strain) (Lys Rif^R), and *E. coli* MG1655 Δ*ara* streptomycin-resistant (susceptible to λ phage, phage-free strain) (Sus Str^R).

The strain *E. coli* MG1655 Δ*ara* λ^+ streptomycin-resistant was obtained from *E. coli* MG1655 Δ*ara* and λ phage as follows. First we obtained the lysogen and only then we isolated a spontaneous streptomycin-resistant clone. To obtain the *E. coli* MG1655 Δ*ara* λ^+ strain we added 0.1 ml of an overnight culture of the *E. coli* MG1655 strain to a tube of molten LB soft agar (LB with 7.5 g/l of agar), mixed, and poured it onto a plate of agar (15 g/l). Then, we put 5 μl of a stock of the "Papa" strain of λ phage (from CBS-Knaw - The Netherlands Culture Collection of Bacteria – NCCB3442). After 15 minutes (to allow for polymerization of the agar), we incubated the plate at 37 ºC. The next day, we obtained a turbid plaque. The turbidity is caused by the growth of cells that are immune (lysogens) or resistant to λ phage. We then purified bacterial clones that are not affected by λ phage and tested these clones for resistance to λ_{vir} (NCCB 3467). If it is affected by λ_{vir}, it is a lysogenic clone. A clone that is neither affected by λ nor by λ_{vir} is resistant. So, we isolated a clone that is resistant to λ phage but susceptible to λ_{vir}. Then we isolated a spontaneous streptomycin-resistant clone from this lysogenic clone by plating 0.1 ml of an overnight culture of *E. coli* MG1655 Δ*ara* λ^+ in a LB-agar plate with streptomycin (100 μg/ml) and incubated overnight (37 ºC). After inoculation, we streaked one of the clones in a similar plate and incubated overnight (37 ºC). The previous step was repeated once more. Then a single clone was stored at -20 ºC.

The rifampicin-resistant *E. coli* MG1655 Δ*ara* strain was isolated by plating 0.1 ml of an overnight culture of *E. coli* MG1655 Δ*ara* on a LB-agar plate with rifampicin (100 μg/ml) and incubated overnight (37 ºC). The next two days we proceeded as explained before for the streptomycin-resistant strain.

The λ^r rifampicin-resistant *E. coli* MG1655 Δ*ara* was obtained as described for the lysogenic strain, except that we added 5 μl of a stock of λ_{vir} phage (instead of λ phage). As previously explained, a resistant *E. coli* can grow in the presence of this virulent phage (but neither susceptible nor lysogenic *E. coli* cells can grow in its presence).

Preparation of strains with reversed markers: the strain *E. coli* MG1655 λ^+ rifampicin-resistant was obtained from *E. coli* MG1655 and λ phage as follows. We used the spontaneous rifampicin-resistant clone mentioned above (where it was used as susceptible λ-free strain). To obtain a lysogenic rifampicin-resistant *E. coli* MG1655 strain we proceeded as described above for the lysogenic streptomycin-resistant strain. As explained in the beginning of this section, the strain *E. coli* MG1655 Δ*ara* λ^+ streptomycin-resistant was obtained first by obtaining the lysogen and only then we isolated a spontaneous streptomycin-resistant clone. Therefore, to obtain strains with reverted markers, we proceeded as follows. To obtain a λ-free streptomycin-resistant clone isogenic to the streptomycin-resistant *E. coli* MG1655 λ^+ strain, we sequenced the mutation in the *rpsL* gene in that streptomycin-resistant lysogenic strain. Then, we isolated eighteen spontaneous streptomycin-resistant clones from the wild-type *E. coli* MG1655 strain and sequenced the *rpsL* gene in all of them. To obtain the streptomycin-resistant clones we plated 0.1 ml of each of the eighteen overnight cultures of *E. coli* MG1655 on a LB-agar plate with streptomycin (100 μg/ml) and incubated overnight (37 ºC). The next two days we isolated spontaneous resistant clones as explained above for the rifampicin-resistant strain and for the lysogenic streptomycin-resistant clone.

PCR amplification of the *rpsl* gene in each clone was performed with 2 μl of pure bacteria culture added to a 25 μl PCR mixture reaction with 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), 1.25 μM of each primer (Chumpolkulwong, Hori-Takemoto et al. 2004), 1 U of Taq polymerase, 1X reaction buffer and 4mM of $MgCl_2$ (NZYTech company). The temperature profile consists of an initial denaturizing step at 95 ºC (10 min), followed by 35 cycles of 95 ºC (1 min), annealing temperature of 64 ºC (1 min), 72 ºC (1 min), and a final extension step at 72 ºC (10 min). The amplification products were visualized in an agarose gel (2%) stained with Red Safe (iNtRON Biotechnology). The PCR product was extracted from the agarose gel with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced by Sanger sequencing method by the StabVida Company.

2.3.2 Measurement of growth rates of the strains Lys Str^R, Sus Rif^R, Res Rif^R

We placed 10 µl of a pre-culture grown overnight at 37 ºC with agitation (170 rpm), in 10 ml of Luria Broth (LB) medium and incubated at 37 ºC with agitation (170 rpm). We did this in triplicate for each strain: the streptomycin-resistant *E. coli* K12 MG1655 Δ*ara* λ^+, the λ-free susceptible rifampicin-resistant *E. coli* K12 MG1655 Δ*ara* strain, and the λ-resistant (and λ-free) rifampicin-resistant *E. coli* K12 MG1655 Δ*ara* strain. Every 30 minutes, a sample of 500 µl was taken to measure the optical density at 670 nm. Growth rates were determined by linear regression. One-Way ANOVA analysis was performed to compare the growth rates of the different strains.

2.3.3 Fitness of strains Lys Str^R, Sus Str^R, Lys Rif^R and Sus Rif^R

The four strains used are Δara. To measure their relative fitness, we performed competition experiments, in LB medium at 37 ºC with agitation for 24 hours, against a reference strain (unaffected by phage lambda), S*taphylococcus aureus* (Ara^+) in an approximate proportion of 1:1. The strains were previously grown in liquid LB medium for 24 hours at 37 ºC with aeration. The values of the each strain were estimated by plating a dilution of the mixture in TA (short for tetrazolium and arabinose) agar. The relative fitness of each strain was calculated according to the expression: $Log_2[S(t)/S(0)]/ Log_2[R(t)/R(0)]$, where S(t) and R(t) are the final values of the strain assayed and the reference *S. aureus* strain respectively and S(0) and R(0) are the initial values of the same strains.

2.3.4 Experimental Competitions

Competitions between lysogenic and susceptible strains were performed in structured and unstructured habitats. We initiated competitions at different initial ratios of lysogenic to susceptible strain (between 10^4:1 and 1:10^4). Competitions for each initial ratio were made in triplicate.

2.3.5 Competitions in the Unstructured Habitat

We inoculated approximately 10^6 total bacteria (at initial different ratios of lysogenic to susceptible cells) in 10 ml of liquid LB medium and incubated at 37 ºC with shaking (170 rpm). At 24 hours intervals for five days, the cultures were diluted 1000 fold in liquid LB medium and incubated again at 37 ºC and 170 rpm.

On the first, third and fifth days of competition, we screened for the densities of λ phages, of lysogenic cells, susceptible cells, susceptible cells that became resistant to λ phage (i.e., cells with the same marker as susceptible cells but that are no more affected by λ neither by λ_{vir}), and susceptible cells that became lysogenic (i.e., cells with the same marker as susceptible cells that are no more affected by λ phage but that are susceptible to λ_{vir} phage). For that, we performed appropriate dilutions of the mixtures and plated on LA supplemented with streptomycin (100 μg/ml) or rifampicin (100 μg/ml). Phages were collected through filtration (0.22 μm) and its titer was determined (see bellow). The appearance of lysogenic or resistant individuals of the susceptible population was screened by streaking at least 30 colonies across LA plates containing λ or λ_{vir} phages along one diameter of the Petri dish. Colonies that do not grow when in contact with both phages are susceptible. Colonies that are able to grow in the presence of both phages are λ-resistant. A bacterial cell is lysogenic if its growth is inhibited by λ_{vir} but not by λ phages.

2.3.6 Competitions in the Structured Habitat

We inoculated approximately $3x10^6$ total bacteria (at initial different ratios of lysogenic to susceptible cells) in 3 ml of molten LB top agar (3.75 g/l), poured the mixture on a Petri dish containing a basal layer of agar (15g/l) (Chao and Levin 1981) and incubated at 37 ºC. After 24 hours, the upper layer was scraped into 12 ml of $MgSO_4$ 10^{-2}M, well mixed (agitated on vortex for 3 minutes), and diluted tenfold. We then inoculated 200 μl of the suspension in three milliliters of LB top agar. This procedure was repeated for the next four days.

On the first, third and fifth days of competition, we screened for the densities of λ phages, of lysogenic cells, susceptible cells, susceptible cells that became resistant to

λ phage, and susceptible cells that became lysogenic. For that, we performed as explained above for competitions unstructured habitat.

2.3.7 Determination of phage titer

Phages were collected through filtration (0.22 μm) and the titer was determined by spotting 10 μl of each serial dilution of the phage (spot test) on the surface of solidified lawns of *E. coli* MG1655 Δara Rif^R susceptible to the λ phage. After incubating at 37 ºC for a day, the number of phage plaques was determined. The bacterial lawn was prepared by pouring 3 ml of molten LB top agar (3.75 g/l), inoculated with 100 μl of an overnight bacterial culture, onto a Petri dish containing a basal layer of agar (15 g/l). Then, phage plaques were counted.

2.3.8 Control Competitions

In order to isolate the effect of λ-phage in competition experiments, we performed further competitions, this time between lysogenic and resistant strains. The initial ratios lysogenic:resistant were $1:10^6$, $1:10^5$,$1:10^4$, …,$10^6:1$. The ratios of these competitions were measured after 24 and 48 hours. Competitions for each initial ratio were made in triplicate. Details about this procedure, including a mathematical model, are given in the Supporting Material and Methods S1.

2.3.9 Measurement of the density of free λ phage in pure cultures of lysogenic cells

We measured the density of free λ phage in pure cultures of lysogenic cells, both at unstructured and structured habitats as follows. Unstructured habitat: we inoculated approximately 10^6 total bacteria in 10 ml of liquid LB medium. After 24 hours of incubation at 37 ºC with shaking (170 rpm), phages were collected through filtration (0.22 μm) and the titer was determined using the spot-test method. Structured habitat: we inoculated approximately $3x10^6$ total bacteria in 3 ml of molten LB top agar (3.75 g/l). This was then poured on a Petri dish containing a basal layer of agar (15 g/l) and incubated at 37 ºC. After 24 hours, the upper layer was scraped into 12 ml of $MgSO_4$ 10^{-2} M and well mixed. Phages were recovered by filtration and its titer was determined.

2.4 RESULTS

The generation times of the lysogenic streptomycin-resistant strain (mean ± 2xStandard Error = 23.40 ± 1.29 min), of the λ-susceptible rifampicin-resistant strain (24.51 ± 0.79 min) and of the λ-resistant rifampicin-resistant strain (23.69 ± 0.67 min) are not significantly different from each other (one-way ANOVA, d.f.(2,6), p=0.30). The λ-resistant strain was included in this comparison because it has previously been shown that some mutations conferring λ-resistance may change bacterial growth rate (Death, Notley et al. 1993) (Pelosi, Kuhn et al. 2006).

We then performed direct competitions in unstructured habitats (liquid LB medium) and in structured habitats (soft LB agar plates) between the lysogenic and susceptible strains starting at different initial ratios. Lysogenic cells were marked with streptomycin resistance and susceptible cells with rifampicin resistance. Colony forming units of each strain were counted on days 0, 1, 3 and 5 by plating cultures on LB-agar plates supplemented with streptomycin or rifampicin. Figure 1 shows the dynamics of the two bacterial populations in competition during five days.

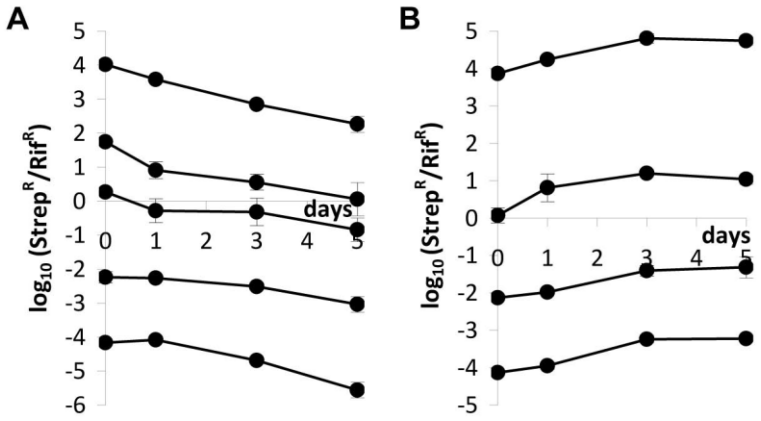

Figure 1. Logarithm of the ratio of streptomycin-resistant to rifampicin-resistant strains during competitions (A) *in unstructured and* ***(B)*** *in structured environments. In the beginning of competitions, streptomycin-resistant cells were lysogenic and rifampicin-resistant cells were susceptible to λ phage. In the unstructured habitat, initial ratios were (lysogens:susceptibles) 1:10^4, 1:10^2, 1:1, 10^2:1, and 10^4:1. In the structured habitat, initial ratios were 1:10^4, 1:10^2, 1:1, and 10^4:1. Each data point represents the mean value of three independent competitions. Error bars represent twice the standard error of the mean (sometimes not seen because they are smaller than data points).*

In the unstructured habitat, the ratios of lysogenic to susceptible cells (streptomycin-resistant to rifampicin-resistant cells) decreased during the five days of competition (Figure 1A; linear regressions tell us that all lines have negative slopes; ANOVA, d.f.=(1,10); $p<1.5x10^{-3}$ for all lines). Therefore, lysogenic cells are at a disadvantage at all initial ratios of lysogenic to susceptible cells (Figure 1A). Because susceptible cells (rifampicin-resistant) have the possibility to become lysogenic or resistant to the λ phage during the five days of competition, we looked for rifampicin-resistant cells that were also resistant or immune to the λ phage (see Materials and Methods) and found no sign of such cells.

In a structured habitat, the bacterial population marked with streptomycin resistance (the only lysogenic cells in the beginning of the competition assay) have an advantage over rifampicin-resistant cells (initially susceptible to λ phage) at all initial ratios of lysogenic to susceptible cells (Figure 1B; regression lines with positive slopes; ANOVA, d.f.=(1,10), $p<8x10^{-3}$ for all lines). However, in the structured habitat, most susceptible cells (rifampicin-resistant) became lysogenic (Figure 2), contrary to what happened in the liquid cultures. Figure 3 shows the ratios of lysogenic to susceptible cells during competitions in the structured habitat, already taking into account the lysogenization of rifampicin-resistant cells.

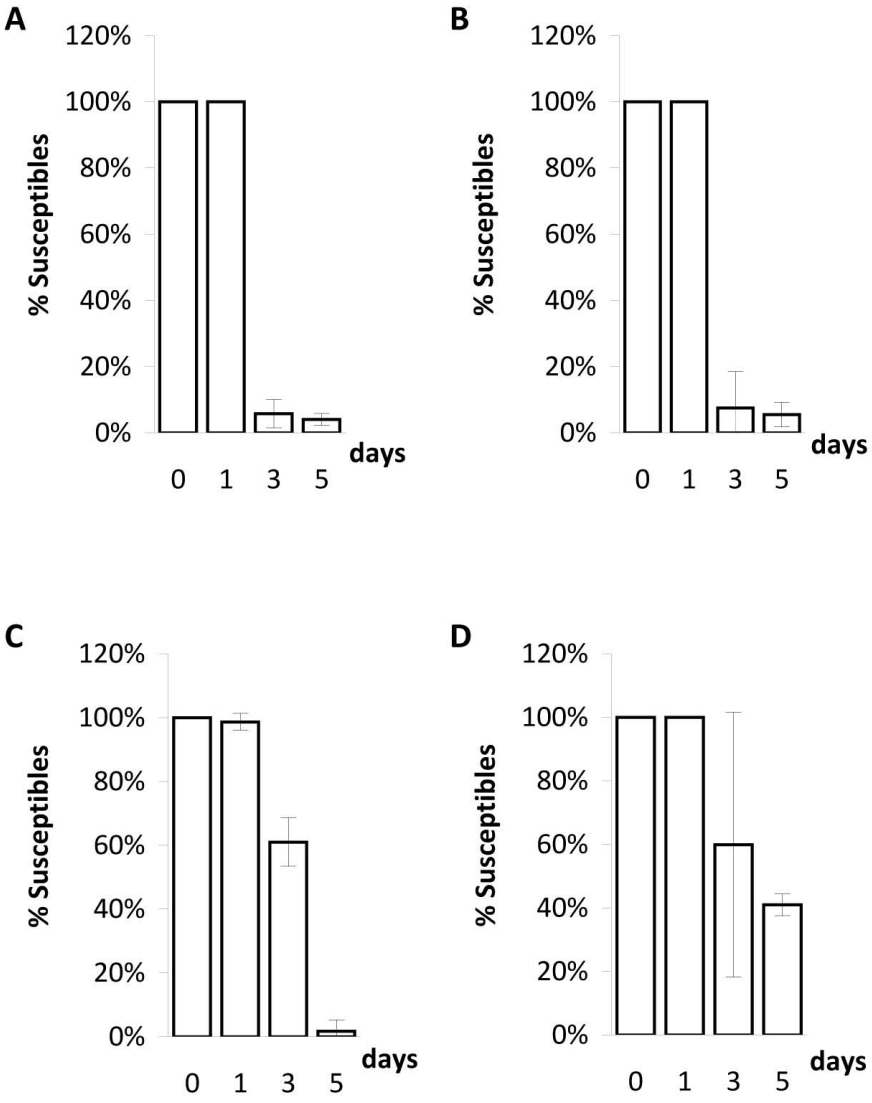

Figure 2. Proportion of susceptible cells among rifampicin-resistant cells during competitions in structured habitats. *Each set of columns represent the mean of the percentage of rifampicin-resistant cells susceptible to λ phage at days 0, 1, 3 and 5. Error bars as in Figure 1. Ratios of lysogenic to susceptible cells as follows:* ***A)*** *ratio = 1:10^4;* ***B)*** *ratio = 1:10^2;* ***C)*** *ratio = 1:1;* ***D)*** *ratio = 10^4:1.*

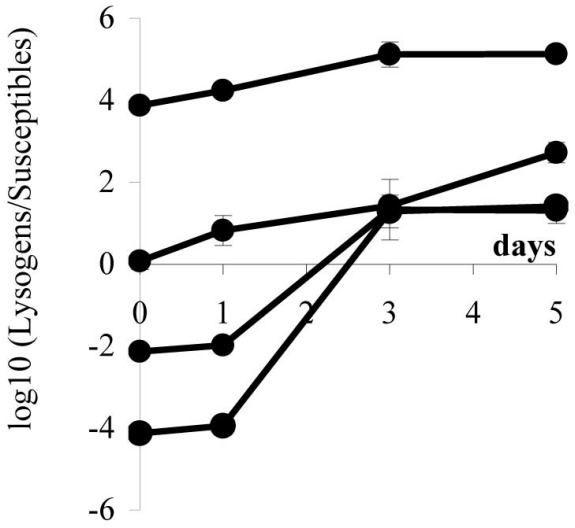

Figure 3. Logarithm of the ratio of lysogenic to susceptible bacteria during competitions in structured environments. *Initial ratios were 1:10^4, 1:10^{-2}, 1:1, and 10^4:1. Lysogenic cells include original lysogenic cells and their descendants (streptomycin-resistant) as well as lysogenized susceptible cells (rifampicin-resistant) and their descendents. Each data point represents the mean value of three independent values. Error bars as in Figure 1*

In Figures 4 (unstructured habitat) and 5 (structured habitat), we show the density of the two competing strains as well as the density of free λ phages in the medium. We also show the expected density of λ phages if lysogenic cells were growing in pure cultures. This value was calculated knowing that, in pure cultures of lysogenic cells, there is a free λ phage per (3.13±0.81)x10^4 lysogenic cells (mean ± twice the standard error). We consider that there is phage amplification among susceptible cells if the density of phages in the competition assay is more than tenfold the typical value found in pure cultures of lysogenic cells, that is, if phage density is 3.13x10^5 phages per ml or more.

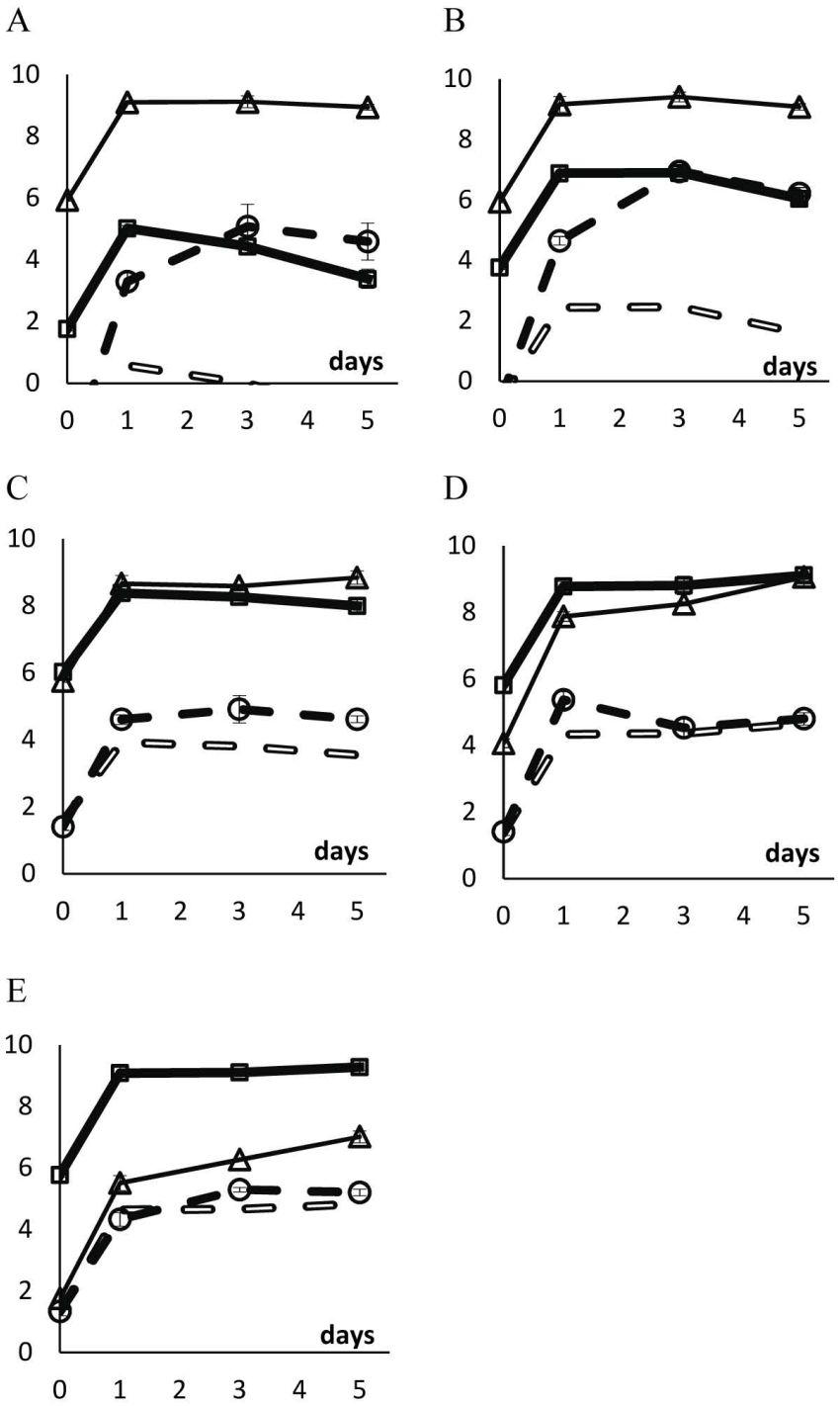

Figure 4.Logarithm of the densities of λ phages and of bacterial strains during competition in the unstructured habitat. Vertical axes represent the $\log_{10}$ of the density of lysogenic rifampicin-resistant cells (thick full line with squares), of susceptible streptomycin-resistant cells (thin line with triangles), of lysogenic streptomycin-resistant cells (dotted line), and of the λ phage (filled broken line with circles), in the beginning of competitions (day 0), and after 1, 3 and 5 days of competition. Error bars as in Figure 1. Open broken lines represent the expected density of λ phages if lysogenic cells were the only cells producing the phage; this is calculated assuming that there is a λ phage per 3.13×10^4 lysogenic cells in pure cultures of lysogenic cells. In all competitions, the initial total bacterial density was around 10^6 cells ml^{-1}. We varied the initial ratio of lysogenic to susceptible cells. **A**: ratio = $1:10^4$; **B**: ratio = $1:10^2$; **C**: ratio = 1:1; **D**: ratio = $10^2:1$; **E**: ratio = $10^4:1$

As mentioned above, in the unstructured habitat, the ratios of lysogenic to susceptible cells decreased between day 1 and day 5 (Figure 1A). However, the cause of such decrease is not the same when the initial ratio is low (Figures 4A and 4B) or high (Figures 4D and 4E). When lysogens are initially rare, their density decreases (ANOVA, d.f.=(1,7); ratio of 10^{-4}: $p<0.0001$; ratio of 10^{-2}: $p<0.01$) whereas the density of susceptible cells is stable (ANOVA, d.f.=(1,7), $p>0.05$). When susceptible cells are initially rare, their density increases (ANOVA d.f.=(1,7); ratio of 10^{4}: $p<0.00001$; ratio of 10^{2}: $p<0.0001$) whereas the density of lysogens is stable (ANOVA, d.f.=(1,7), $p>0.05$).). Interestingly, at low initial ratios (10^{-4} and 10^{-2}), the virus does not impose a discernible cost to susceptible cells. Presumably this is because the density of viruses is very low and the density of susceptible cells is very high. Nevertheless, at these low initial ratios, susceptible cells do play a role as amplifiers of viruses, as virus density is much higher in these competitions than it would be expected if only lysogenic cells were present in the culture.

When the initial ratios of lysogenic to susceptible cells are 10^{0}, 10^{2} and 10^{4}, susceptible cells do not amplify the virus (Figures 4C, 4D and 4E). Moreover, when ratios are 10^{2} or 10^{4}, susceptible cells are able to increase in density between days 1 and 5 (ANOVA, d.f.=(1,7), $p < 0.0001$). One can explain these observations with the fact that the λ virus is also adsorbed by lysogenic cells (but viruses are not able to replicate inside these cells).

In competitions performed in soft agar plates, there is virus amplification in the competition assays in which the ratio of lysogenic to susceptible cells is initially close to 10^{-4}, 10^{-2}, or 10^{0} (Figures 5A, 5B and 5C). Indeed, in these competition experiments, and between days 1 and 5, the density of free viruses is higher than tenfold the density of viruses when lysogenic cells are growing in a pure culture (i.e., in the absence of susceptible cells). However, there is no amplification of viruses by susceptible cells when the ratio of lysogenic to susceptible cells is initially 10^{4} (Figure 5D). This was expected given that, in these competitions (Figure 5D) the density of susceptible cells is much lower than in the previously mentioned competitions (Figures 5A, 5B and 5C).

In the structured habitat, the density of susceptible cells decreases at all initial ratios (ANOVA d.f.=(1,7); ratio of 10^{-4}: $p<0.01$; ratio of 10^{-2}: $p<0.05$; ratio of 10^{0}: $p<0.0004$; ratio of 10^{4}: $p<0.02$). The density of lysogenic cells, however, increases when

initially rare (Figure 5A and 5B; ANOVA d.f.=(1,7); ratio of 10^{-4}: $p<0.003$; ratio of 10^{-2}: $p<0.005$) and is stable when ratios are 10^0 and 10^4 (Figure 5C and 5D; ANOVA d.f.=(1,7); $p>0.05$).

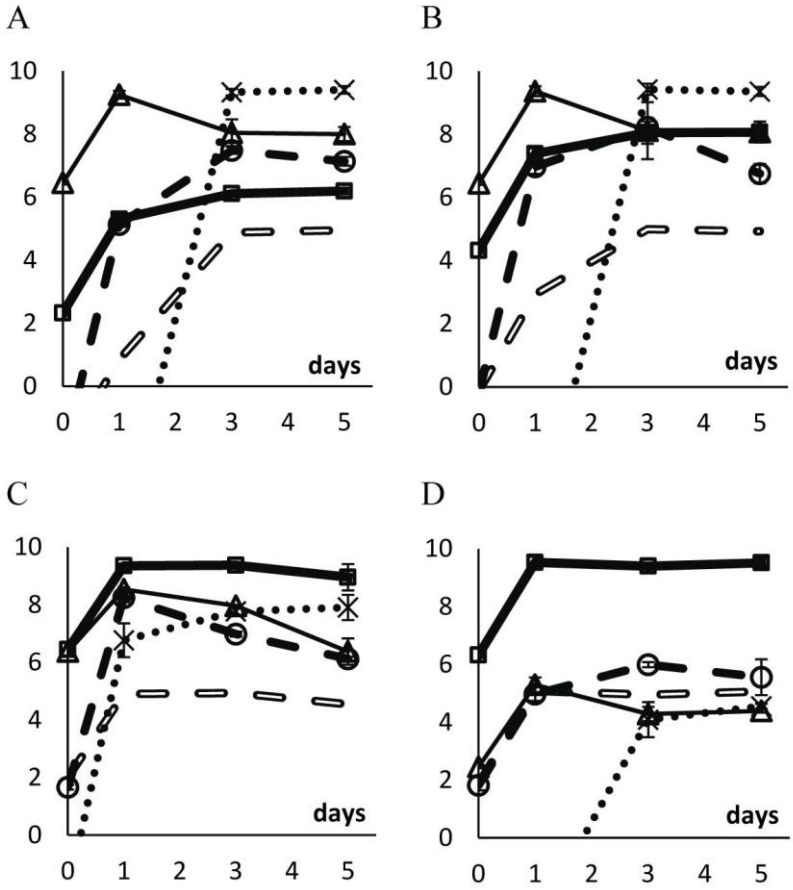

Figure 5. Logarithm of the densities of λ phages and bacterial cells during competitions in the structured habitat. *Vertical axes represent the log_{10} of the density of lysogenic rifampicin-resistant cells (thick full line with squares), of susceptible streptomycin-resistant cells (thin line with triangles), of lysogenic streptomycin-resistant cells (dotted line), and of the λ phage (filled broken line with circles), in the beginning of competitions (day 0), and after 1, 3 and 5 days of competition. Error bars as in Figure 1. Open broken lines represent the expected density of λ phages if lysogenic cells were the only cells producing the phage; this is calculated assuming that there is a λ phage per $3.13x10^4$ lysogenic cells in pure cultures of lysogenic cells. In all competitions, the initial total bacterial density was around $3x10^6$ cells ml^{-1} of soft LB agar medium. We varied the initial ratio of lysogenic to susceptible cells.* ***A****: ratio = $1:10^4$;* ***B****: ratio = $1:10^2$;* ***C****: ratio = 1:1;* ***D****: ratio = $10^4:1$.*

In order to isolate the effect of λ phage in competition experiments, we reasoned as follows. Two main factors may have an impact on the values of the Str^R/Rif^R ratios: the λ phage and chromosomal markers. Therefore, we performed competition experiments, this time where rifampicin-resistant cells are resistant to the λ phage. In these experiments, the initial ratios Str^R/Rif^R were 10^{-6}, 10^{-5},…, 10^6. The ratios of the

density of each strain were measured for periods of 24 and 48 hours (the same time periods used in Figure 1). Then, we calculated the expected density of lysogenic cells if antibiotic-resistance markers had no effect (see Supporting Material and Methods S1 for a mathematical explanation). This was possible because we compared the growth rates of these three strains (lysogenic (Str^R), λ-susceptible (Rif^R) and λ-resistant (Rif^R)) and they are similar, as stated before. With this method we isolated the effect of λ phage during competitions. By comparing Figure 1 with Figure 6 one can see that, while these markers indeed affect our competition assays, they do not change our major conclusions.

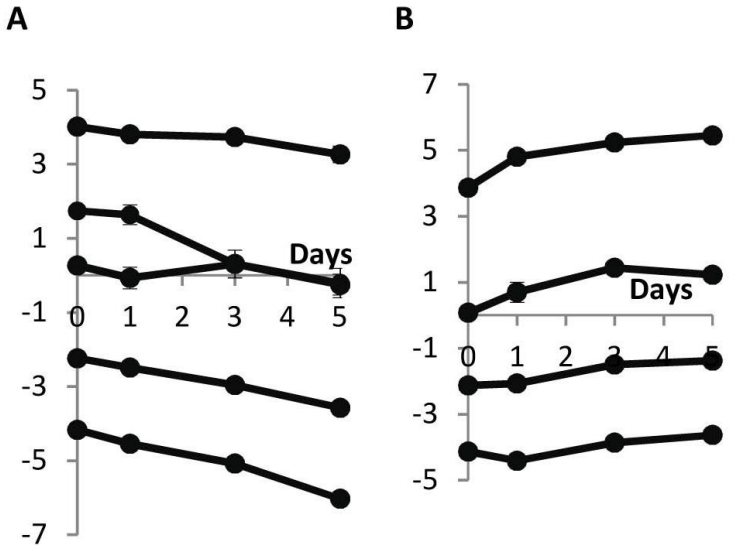

Figure 6. Logarithm of the ratio of expected densities of lysogenic streptomycin-resistant to λ-resistant rifampicin-resistant cells (A) *in unstructured and* ***(B)*** *in structured environments. Error bars as in Figure 1.*

As just mentioned, the distinction between the strains involved in the competition experiments was possible due to genetic markers (at *rpoB* and *rpsL* genes) conferring antibiotic resistance. So, what would happen if competition experiments were performed with reverted markers?

Unfortunately, mutations in the *rpoB* and *rpsL* genes can affect the viral development of λ phage. Different mutations in the *rpo*B gene have distinguished effects on the phage development (for a review, see ref. (Friedman, Olson et al. 1984)). For example, mutation *rpo*B111 (Jin and Gross 1989) restricts the growth of phage λ, while mutation *rpo*B501 (Lecocq and Dambly 1976) decreases the frequency of

lysogenization and lysis is delayed because the endolysin synthesis is reduced. Mutations in the *rps*L gene (encoding the ribosomal protein S12) can restrict the synthesis of viral proteins and/or decrease the efficiency of translational initiation, even though the degree of restriction varies according to the mutation (Yates, Gette et al. 1977, Li, Weng et al. 1998). Therefore, the interactions when a given rifampicin-resistant cell is the lysogen may be different from the interactions when the rifampicin-resistant strain is the recipient of the lambda phage (susceptible host). The same reasoning applies for streptomycin-resistant cells. Therefore, it is not certain that, in competition experiments, a rifampicin-resistance lysogen behaves exactly like a streptomycin-resistant lysogen. Similarly, it is not certain that a rifampicin-resistant susceptible host cell behaves like a streptomycin-resistant host cell. The implication of this is that one may observe a higher or lower level of phage amplification and/or lysogenization rate with other chromosomal markers – which includes the situation in which the markers are reversed, as is explained above. In a wider perspective, and given previous works concerning the study of epistasis when mutations at the *rpoB* and *rpsL* are involved (Trindade, Sousa et al. 2009, Silva, Mendonca et al. 2011, Breen, Kemena et al. 2012), epistasis between these mutations and λ-phage are indeed expected. Because of these effects, competition experiments between lysogenic and susceptible strains with reverted markers are not expected to give the same result as with the original markers. To check this, we performed competition experiments for initial ratios of 10^{-4} and 10^{4} in structured and unstructured media. Before that, however, we compared the fitness of the strains that were constructed to performed competitions (with original and reverted markers), and we did not find significant differences (one-way ANOVA, d.f.=(3,16), p=0.06). Then, with reverted markers, we performed competitions between lysogens and susceptible strains.

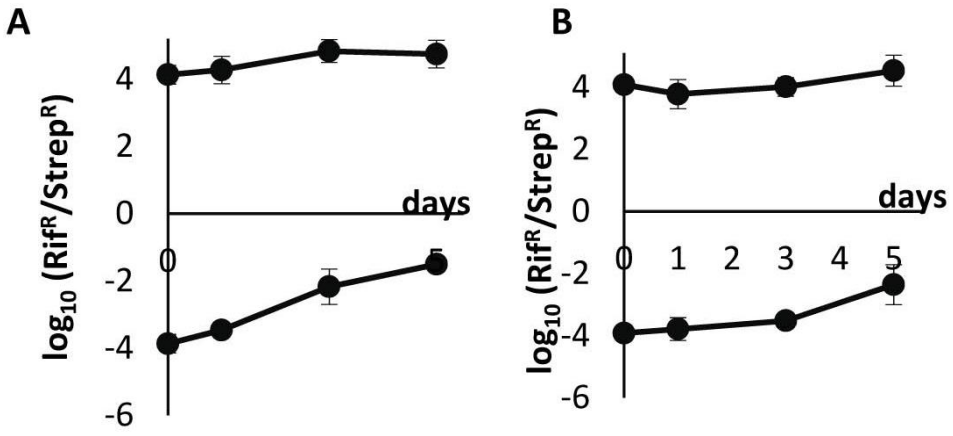

Figure 7. The same as Figure 1, *this time with exchanged markers. Logarithm of the ratio of rifampicin-resistant to streptomycin-resistant strains during competitions* ***(A)*** *in unstructured and* ***(B)*** *in structured environments. In the beginning of competitions, rifampicin-resistant cells were lysogenic and streptomycin-resistant cells were susceptible to* λ *phage. In both habitats, initial ratios were* $1:10^4$*, and* $10^4:1$ *Each data point represents the mean value of three independent competitions. Error bars as in Figure 1.*

In Figure 7A one can see that there was no disadvantage for lysogenic cells in the unstructured environment (contrary to what we observe in Figure 1A). Since the fitnesses of the strains are similar, differences between Figures 1A and 7A are not due to costs imposed by the antibiotic-resistance markers *per se*, but by interactions between the host mutations and the phage genetic system.

In Figure 7B, one can see that, in structured habitat, results obtained with reverted markers are qualitatively similar to the ones obtained previously (Figure 1B).

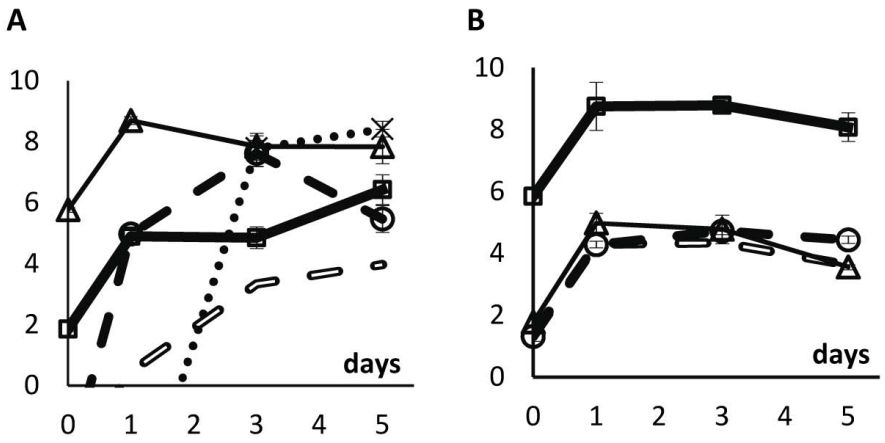

Figure 8. Logarithm of the densities of the λ phage and bacterial strains populations during competition in the unstructured habitat with reversed markers. Vertical axes represent the $\log_{10}$ of the density of lysogenic rifampicin-resistant cells (thick full line with squares), of susceptible streptomycin-resistant cells (thin line with triangles), of lysogenic streptomycin-resistant cells (dotted line), and of the λ phage (filled broken line with circles), in the beginning of competitions (day 0), and after 1, 3 and 5 days of competition. Many streptomycin-resistant cells became λ-resistant already at days 3 and 5 (data not shown) in this competition with densities similar to the ones reached by lysogenic streptomycin-resistant cells. Error bars as in Figure 1. Open broken lines represent the expected density of λ phages if lysogenic cells were the only cells producing the phage; this is calculated assuming that there is a λ phage per 3.13×10^4 lysogenic cells in pure cultures of lysogenic cells. In all competitions, the initial total bacterial density was around 10^6 cells ml^{-1}. A: initial ratio of lysogenic to susceptible cells = $1:10^4$; B: initial ratio of lysogenic to susceptible cells = $10^4:1$.

Figures 8 and 9 show more details of competitions performed in unstructured and structured media with reverted markers. In structured media results with original and reverted markers are similar (compare Figures 5 and 9), as well as in liquid when the initial frequency is 10^4 (compare Figures 4E and 8B). However, in liquid media, when the initial frequency was 10^{-4} , results are considerably different, because (i) the appearance of lysogens among the initial susceptible cells (near 10^8 lysogens per ml after 3 days only, Figure 8A), which did not happen with original markers (Figure 4A); (ii) therefore, levels of phage in the medium are much higher (compare Figures 4A and 8A); (iii) hence selecting for the appearance of λ-resistant and lysogenic cells among the susceptible streptomycin-resistant cells (near 10^8 lysogens per ml after 3 days only, something that did not happen with original markers).

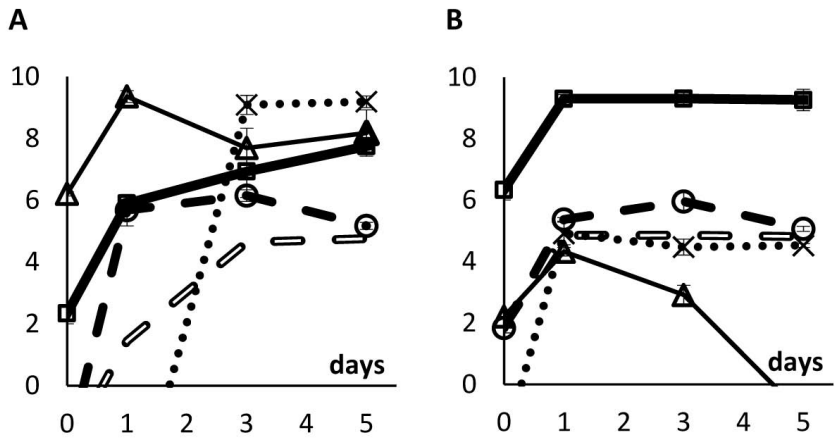

Figure 9. Logarithm of the densities of λ phages and of bacterial strains during competition in the structured habitat with reversed markers. Vertical axes represent the $\log_{10}$ of the density of lysogenic rifampicin-resistant cells (thick full line with squares), of susceptible streptomycin-resistant cells (thin line with triangles), of lysogenic streptomycin-resistant cells (dotted line), and of the λ phage (filled broken line with circles), in the beginning of competitions (day 0), and after 1, 3 and 5 days of competition. Error bars as in Figure 1. Open broken lines represent the expected density of λ phages if lysogenic cells were the only cells producing the phage; this is calculated assuming that there is a λ phage per 3.13×10^4 lysogenic cells in pure cultures of lysogenic cells. In all competitions, the initial total bacterial density was around 10^6 cells ml^{-1}. **A**: initial ratio of lysogenic to susceptible cells = $1:10^4$; **B**: initial ratio of lysogenic to susceptible cells = $10^4:1$.

2.5 DISCUSSION

In unstructured media, secreted molecules from a few bacterial cells are expected to be equally spread all over the liquid volume, which may imply that the secreted molecule is at low concentration in the full volume. However, in structured media, excreted molecules are likely to be concentrated in the vicinity of producer cells and to be almost totally absent in the rest of the media.

Now suppose that the excreted substance has an antagonistic effect (e.g., a toxin). If higher concentrations of these molecules imply higher antagonistic effects, the antagonistic effect is predicted to be higher in structured than in unstructured habitats simply because, in this case, the concentration of the molecule is high in the neighbourhood of producers. Therefore, unless the production of toxins is extremely high, one may expect that the outcome of competition experiments between toxin producers and sensitive cells is frequency dependent in which producer cells are at an advantage only when fairly common (Chao and Levin 1981).

If the "toxic substance" is a temperate bacteriophage, the dynamics of the system becomes more interesting because the virus may replicate inside their victims just before killing them (Brown, Le Chat et al. 2006, Dionisio 2007). However, the outcome of competition is more difficult to predict than in the case of toxins because, with temperate phages, there is the possibility of lysogenization. Finally, at least in the case of λ phage, predictions are even more complicated because there is a positive correlation between λ phage concentration in the medium and the probability of lysogenization (Kourilsky 1973, Kourilsky and Knapp 1974, Zeng, Skinner et al. 2010).

Given these opposing forces, we asked whether λ phages can be used by their hosts as biological weapons as an alternative to toxins. For that, competition experiments between lysogenic cells and susceptible cells were performed.

In liquid cultures there is phage-amplification when lysogens are initially rare: this observation arises from the fact that there are many more phages in the medium than the number of expected phages if lysogens were alone (Figures 4A, 4B, and 8A). In liquid culture, with reverted chromosomal markers, we also observed lysogenization at days 3-5 (Figure. 8A) – probably because, already at day 1, the density of phages was 100-fold higher with reverted markers (around10^5 phages/ml) than with the original

markers (around 10^3 phages/ml). It is interesting to note these differences with different markers as they suggest the presence of epistasis between λ-genes and the chromosomal markers at *rpsL* or *rpoB* genes.

In the structured habitat there is phage amplification in most conditions (Figure. 5 and Figure. 9), and most susceptible cells became lysogenic, hence immune to the phage (Figure.5 and Figure. 9).

Given these results we conclude that bacteriophages indeed behave as allelopathic agents because the susceptible population can suffer from the impact of phages in both types of habitat. However, this is a transient advantage because, in our experiments, lysogenization of susceptible cells occurs rapidly. This would not be possible with toxins (unless, of course, they were coded in mobile elements such as conjugative plasmids). In other words, although phages act as efficient allelopathic agents, the immunity genes are also offered to competitors through lysogenization (with the exception of low frequency of lysogens in liquid media where amplification was not followed by lysogenization).

As explained above, one should certainly expect higher levels of lysogenization in structured habitats than in a liquid environment because lysogeny is favoured when many phages enter into the same cell at once (Kourilsky 1973, Kourilsky and Knapp 1974, Zeng, Skinner et al. 2010). Indeed, while in unstructured environments, the ratio of the density of λ phages to bacterial cells usually takes a very low value, in structured environments, the proportion of lysogenic to susceptible cells at a local scale may be radically different from the value at a global scale. Therefore, in the context of a structured habitat, one has to consider local interactions.

Some previous studies report similar experiments on both structured habitats and unstructured habitats. Bossi and colleagues tested the contribution of gifsy-1 and -2 viruses to the dynamics of *Salmonella enterica* serovar Typhimurium bacterial populations. The authors performed competitions in static liquid cultures between infected and uninfected bacterial cells. They showed that spontaneous virus induction in a few lysogenic cells enhanced the competitive fitness of the lysogen population as a whole (Bossi, Fuentes et al. 2003). Similarly, and using competitions in liquid cultures, Joo et al. showed that *Bordetella bronchiseptica* bacterial cells use BPP-1 temperate viruses to mediate competition with other *B. bronchiseptica* cells (Joo, Gunny et al.

2006). Later, Erickson et al. observed that archived cultures of *S. enterica* serovar Typhimurium in competition in soft agar plates gained some selective advantage over non-archived cultures of *S. enterica* because a small proportion of archived bacterial cells yielded the virus fels-1 and -2 which lysed non-archived cultures of *S. enterica* (Erickson, Newman et al. 2009). In 2006, Brown et al. compared the impact of the ϕ80 temperate viruses with that of toxins (bacteriocins) in liquid cultures and found that the former behaved as "replicating toxins" for three days. By performing experiments with different ratios of lysogenic to susceptible cells, the authors have shown that, in an unstructured habitat, bacteriophage ϕ80 is mostly useful in the context of invader offense (that is, when lysogens compete with a resident bacterial community), rather than chemical toxins (bacteriocins) that are favoured in the context of resident defence (Brown, Le Chat et al. 2006).

Some of these previous works indeed show that some viruses seem to be used by bacterial populations as functional equivalents to toxins. However, while most toxins are not expected to be effective in liquid media if toxin-producers are rare, bacteriophages may be efficient weapons (Figure. 4, Figure. 8 and references. (Brown, Le Chat et al. 2006, Joo, Gunny et al. 2006)). Interestingly, it is precisely phage amplification that solves this apparent paradox. In unstructured habitats, each lysogenic cell may interact with most susceptible cells in the liquid medium. If there is amplification of the phage (something impossible with a toxin coded in the chromosome or in a non-mobilizable plasmid) and burst size is 10^2, then five rounds of phage infection and replication would be enough to reach 10^{10} phages, hence killing most or all susceptible cells (Dionisio 2007). Most likely, this is what happened in previous works performed in unstructured habitats (Brown, Le Chat et al. 2006, Joo, Gunny et al. 2006) and partly in our results (Figure. 4B and Figure. 4A).

The experiments shown here were performed with the PaPa strain of λ phage, which lacks side tail fibers (Hendrix and Duda 1992). Due to this characteristic, this phage propagates better in structured environments than strains with side tail fibers, such as the Ur-λ (Gallet, Shao et al. 2009). Therefore it would be interesting to perform similar experiments with Ur-λ phages because they adsorb better to bacterial cells than λ-PaPa phages, despite having less ability to diffuse in structured environments – hence, Ur-λ phages propagate better in liquid than in structured habitats (Gallet, Shao et al. 2009).

Though for a few tens of generations only (before massive lysogenization occurs), we conclude that, indeed, bacterial cells may use bacteriophages as replicating toxins. Despite the risk that phages join competitor bacterial cells, hence not being trustworthy weapons, they may still be extremely useful because, a few days may be enough for lysogens to invade and establish in a new habitat (Brown, Le Chat et al. 2006).

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2.8 SUPPLEMENTARY INFORMATION

2.8.1 Isolating the effect of λ-phage in competition experiments

As explained in the main text, we performed competitions between lysogenic (Str^R) and λ-resistant (Rif^R) strains. The ratios Str^R/Rif^R were $1:10^6$, $1:10^5$,…, $10^6:1$. The ratios of the density of each strain resulting from these competitions were measured after 24 and 48 hours. Then, we calculated the expected density of lysogenic cells if antibiotic-resistance markers had no effect.

Let $\omega_{A/B}$ be the fitness of strain A relative to strain B:

$$\omega_{A/B} = \frac{\log\left(\frac{A_f}{A_i}\right)}{\log\left(\frac{B_f}{B_i}\right)}$$

Now, let $\omega_{\lambda^+,Str^R/\lambda^S,Rif^R}$ be the fitnesses of lysogenic cells (which are Str^R) relative to susceptible cells (Rif^R) and $\omega_{\lambda^+,Str^R/\lambda^R,Rif^R}$ be the fitness of lysogenic cells (Str^R) relative to λ-resistant cells (Rif^R). Then, to first order, one can calculate the fitness of lysogenic cells relative to isogenic susceptible cells (they are both Rif^R) as:

$$\omega_{\lambda^+/\lambda^S} = \omega_{\lambda^+,Str^R/\lambda^S,Rif^R} / \omega_{\lambda^+,Str^R/\lambda^R,Rif^R} \qquad \text{(Equation S1)}$$

The term $\omega_{\lambda^+,Str^R/\lambda^S,Rif^R}$ is the actual value of the competitions shown in Figure 1. As explained above, we measured $\omega_{\lambda^+,Str^R/\lambda^R,Rif^R}$. We measured these values for intervals of one day and intervals of two days because in competition experiments (Figure.1-5), intervals between measurements are of one or two days. We measured these values by performing competition experiments between lysogenic (Str^R) and λ-resistant (Rif^R) strains for intervals of one day and two days. With this process, we obtained an array of values of $\omega_{\lambda^+/\lambda^S(1day)}$ and $\omega_{\lambda^+/\lambda^S(2days)}$.

If we had performed direct competitions between lysogenic cells and susceptible cells (that is, with no chromosomal markers), the fitness $\omega_{\lambda^+/\lambda^S}$ would be calculated as:

$$\omega_{\lambda^+/\lambda^S} = \frac{\log\left(\frac{L_f}{L_i}\right)}{\log\left(\frac{S_f}{S_i}\right)} \qquad \text{(Equation S2)}$$

Note that the values for $\omega_{\lambda^+/\lambda^S}$, are $\omega_{\lambda^+/\lambda^S(1day)}$ and $\omega_{\lambda^+/\lambda^S(2days)}$. Therefore, putting together Equations S1 and S2, the density of lysogens after competition would be given by:

$$L_f = L_i\left(\frac{S_f}{S_i}\right)^{\omega_{\lambda^+/\lambda^S}} \qquad \text{(Equation S3)}$$

As a result, $L_{1(expected)}$, $L_{3(expected)}$, and $L_{5(expected)}$ are given by:

$$L_{1(\exp ected)} = L_0\left(\frac{S_1}{S_0}\right)^{\omega_{\lambda^+/\lambda^S}(1day)}$$

$$L_{3(\exp ected)} = L_1\left(\frac{S_3}{S_1}\right)^{\omega_{\lambda^+/\lambda^S}(2days)}$$

$$L_{5(\exp ected)} = L_3\left(\frac{S_5}{S_3}\right)^{\omega_{\lambda^+/\lambda^S}(2days)}$$

One can now plot these values (Figure 6 in the main text).

CHAPTER III:

3 SOCIAL BEHAVIOUR INVOLVING DRUG RESISTANCE: THE ROLE OF INITIAL DENSITY, INITIAL FREQUENCY AND POPULATION STRUCTURE IN SHAPING THE EFFECT OF ANTIBIOTIC RESISTANCE AS A PUBLIC GOOD

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Iolanda Domingues, João Gama, Luís Carvalho and Francisco Dionísio

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3.1 ABSTRACT

Bacteria sometimes cooperate with co-inhabiting cells. Pathogenic bacteria, for example, often produce and excrete virulence factors, eventually benefitting both producer and non-producer cells. The role of social interactions involving antibiotic resistance, however, has been more elusive. Enzymes that inactivate β-lactam antibiotics such as ampicillin or penicillin (β-lactamases) are good candidates as public goods. Nonetheless, it has been claimed that bacteria harbouring plasmids of natural origin coding for β-lactamase almost do not protect sensitive bacteria. This does not fit with the fact that ampicillin-sensitive bacteria can be isolated from subjects undergoing ampicillin treatment. We hypothesized that there are two non-exclusive explanations for the discrepancy between previous works: (1) the range of values of demographic conditions (such as initial strain frequency, initial total cell density, or habitat structure) has not been broad enough to include most scenarios, or, (2) there are interactions between some of these factors. We performed experiments with *Escherichia coli* bacterial cells to measure the degree of protection of sensitive cells when co-cultured with cells harbouring RP4, R16a or the R1 plasmids, all of natural origin and coding for β-lactamase, and in presence of ampicillin. In these co-cultures, performed in structured and non-structured environments, both the initial total cell density and the initial frequency of sensitive cells spanned four orders of magnitude. We found protection of sensitive cells in 63% of tested conditions. All factors (plasmid, structure, frequency, and density) significantly affect levels of protection. Moreover, all factors interact, with interactions revealing large or very large effect sizes.

3.2 INTRODUCTION

Cooperative behaviour has been observed in the bacterial world, notably in processes involving pathogenicity (West *et al*, 2007; West *et al*, 2006). A common form of cooperation in bacterial populations is the production of public-goods, metabolically costly molecules produced by one individual that become available to neighbouring individuals (West *et al*, 2007; West *et al*, 2006). Different molecules may act as public goods, ranging from products responsible for scavenging, motility and communication, to substances involved in allelopathy or virulence (West *et al*, 2007).

There are three main mechanisms of antibiotic resistance: pumping out the antibiotic from the cell, inhibiting the interaction of the drug with the target, or by modifying or degrading the antibiotic molecule (Nicoloff and Andersson, 2016; Sorg *et al*, 2016; Wright, 2005). Enzymes that modify the antibiotic may be considered public goods because, by decreasing antibiotic concentration in the local environment, they may protect nearby bacteria. In this study, we focus on the case of β-lactamase enzymes, which degrade antibiotics like ampicillin or penicillin.

Degrading enzymes may detoxify the surrounding environment even if they remain within the cell – for example, in Gram-negative bacteria cells, β-lactamase locates primarily at the periplasmic space (Livermore, 1995), a space located between the inner and outer membranes. This phenomenon will possibly be stronger than it seems because molecules of β-lactamase may escape the periplasm, albeit by leakage rather than by secretion (Livermore, 1995).

The ability of β-lactam sensitive cells to survive when located in the vicinity of β-lactamase producer cells has been noticed several times by molecular biologists in the process of gene cloning, by observing the presence of the so-called “satellite colonies” (Sambrook and Russell, 2001). These small colonies, composed by ampicillin-sensitive bacteria, are able to grow in ampicillin-enriched agar plates due to their proximity to β-lactamase-producing cells. This phenomenon has also been hypothesized to occur in polymicrobial infections, since ampicillin-sensitive bacteria were isolated from subjects undergoing ampicillin treatment (Brook, 2004). More recently, Dugatkin and colleagues

showed that a β-lactamase enzyme, encoded on an artificial plasmid, more specifically on a gene that was modified to inactivate ampicillin outside the cell (and not in the periplasmic space), could indeed protect nearby cells from death (Clark *et al*, 2009; Dugatkin *et al*, 2005; Perlin *et al*, 2009). Furthermore, Gore and colleagues (Yurtsev *et al*, 2013) showed that *E. coli* strains harbouring artificial plasmids coding for non-modified β-lactamases could detoxify the environment hence protecting otherwise ampicillin-sensitive cells from β-lactam antibiotics.

More recently, Medaney and colleagues (Medaney *et al*, 2016) also tested for protection mediated by a β-lactamase enzyme, this time with a natural isolated plasmid (called pCT). Using a natural plasmid is important because it elucidates whether protection of sensitive cells previously observed using artificial plasmids (Yurtsev *et al*, 2013), sometimes even artificially increasing the secretion of β-lactamase (Dugatkin *et al*, 2005), can occur in more natural settings. Using a plasmid of natural origin, however, Medaney et al achieved a different conclusion, observing that sensitive cells do not typically benefit from the presence of resistant cells (Medaney *et al*, 2016). Rather, the authors showed that the few sensitive cells allowed to form colonies are those undergoing a state of persistence, in which bacteria stop dividing (Balaban *et al*, 2004; Lewis, 2010). Such delay in cell division saves those few sensitive cells from death if it provides the time required for antibiotic degradation or direct detoxification by β-lactamase-producing cells (Medaney *et al*, 2016).

In one hand, studies involving artificial plasmids consistently demonstrate the ability of β-lactamase producer cells to protect sensitive cells, unlike the study involving a plasmid of natural origin. A possible explanation for the lack of protection with the pCT plasmid could be that many relevant demographic conditions were not examined, as their experiments focused on very narrow intervals of initial frequencies of plasmid-bearing and plasmid-free cells and of initial total cell densities of both cell strains (Medaney *et al*, 2016).

Indeed, Ross-Gillespie and colleagues have shown the importance of demographic conditions in the exploitation of public goods production among bacteria (Ross-Gillespie *et al*, 2007) (Ross-Gillespie *et al*, 2009). They used the production of siderophores (molecules that scavenge iron from the environment) as a model of cooperative trait. In

a structured environment, non-producers of siderophores, or cheaters, are better able to exploit the cooperative individuals at higher initial total population density – positive density-dependence (Ross-Gillespie *et al*, 2009). In an unstructured environment, there is negative frequency dependence, i.e., non-producers of siderophores have an advantage when rare (Ross-Gillespie *et al*, 2007).

To our knowledge, there is no experimental study of the conjoint effect of different combinations of demographic conditions (such as habitat structure, total cell density and cell frequencies) on the cooperative production of β-lactamase. Through the testing of several combinations one may test for interactions between factors, e.g. between initial total cell density and initial relative strain frequency, a complication expected to occur (Ross-Gillespie *et al*, 2009). For example, in a liquid environment, if the initial total cell density is low, the number of β-lactamase producers may be insufficient to detoxify the environment, eventually leading to the death of all sensitive cells irrespectively of the initial frequencies of sensitive and producer cells – in this case we would not observe frequency dependence. On the other hand, if density is above a certain value, the fitness of the sensitive population is expected to depend on the frequency of producers and sensitive cells.

Our work aims to study the ability of ampicillin-sensitive cells to thrive in environments with lethal doses of ampicillin, focusing on the antibiotic protection conferred by resistant bacteria harbouring three plasmids of natural origin. Using three different plasmids, we perform a systematically study of the levels of protection under different combinations of demographic conditions – population structures, initial strain frequencies, and initial total cell densities – as well as the interactions between these factors.

3.3 MATERIALS & METHODS

3.3.1 Bacterial Strains

In this work we used two isogenic strains of *Escherichia coli,* the wild-type *E. coli* K12 MG1655 and *E. coli* K12 MG1655 *Δara Val*^{R}. The latter strain harbours two chromosomal markers: *Δara* – deletion of the arabinose operon which disables the metabolism of arabinose monosaccharide; and *Val*^{R} – a phenotype that results from a mutation that enables the growth in the presence of valine (40 μg L^{-1}) when isoleucine is absent.

Conjugative plasmids R1, R16a and RP4, of natural origin, were inserted into *E. coli* K12 MG1655 *Δara Val*^{R}*,* through bacterial conjugation, resulting in the three ampicillin resistant strains: *E. coli* K12 MG1655 *Δara Val*^{R} (R1), *E. coli* K12 MG1655 *Δara Val*^{R} (R16a) and *E. coli* K12 MG1655 *Δara Val*^{R} (RP4). All three β-lactamase producing strains are identical (except for the different plasmids) and will therefore be mentioned solely according to the harboured plasmid. The wild-type strain will be mentioned henceforth as the ampicillin sensitive strain.

3.3.2 Experimental conditions of co-culture

The sensitive strain was co-cultured with an ampicillin resistant strain, in a set of four different conditions: (i) ampicillin resistant strain containing one of the three β-lactamase producing plasmids; (ii) two different habitat structures – Structured (solid medium) and Unstructured (liquid medium with constant agitation); (iii) three initial total cell densities – High (10^{7} cells ml^{-1}), Intermediate (10^{5} cells ml^{-1}) and Low (10^{3} cells ml^{-1}); and (iv) three initial frequencies of sensitive cells – high frequency (99S:1R), intermediate frequency (50S:50R) and low frequency of sensitive cells (1S:99R), where S stands for sensitive and R for resistant.

Each condition of co-culture was performed in parallel, both in media containing ampicillin (100 μg ml^{-1}) and in ampicillin-free media. All co-cultures were performed in triplicate.

Additionally, sensitive and resistant strains were cultured in isolation in media containing ampicillin to confirm the ability of the resistant strains to grow and that the sensitive strain don't. This control was performed both in structured and unstructured media, and at all initial total cell densities. After a 24 hours period of incubation, cultures were plated on selective media, and incubated for 48 hours; next steps are described in following two sections: for unstructured and structured media.

3.3.3 Co-cultures in Unstructured Media

The sensitive and ampicillin-resistant strains were inoculated, in each of the appropriate combinations of initial total cell density and strain frequency, in 10 mL of Luria broth (LB) and incubated at 37ºC with constant agitation (170 rpm). We allowed the bacteria to grow in co-culture for 24 hours (reaching the stationary phase).

After incubation, samples of the co-cultures were retrieved, appropriately diluted and plated on selective media for 48h: M9 Minimal Medium with 3 mM $MgSO_4$, supplemented with 4 g L^{-1} of arabinose to select the sensitive strain; and supplemented with 4 g L^{-1} of glucose, 40 μg ml^{-1} of valine, and 100 μg ml^{-1} of both ampicillin and kanamycin to select ampicillin resistant strains. Additionally, all samples were plated on M9 Minimal Media with 3 mM $MgSO_4$, supplemented with 4 g L^{-1} of arabinose, 40 μg ml^{-1} of valine, and 100 μg/mL of both ampicillin and kanamycin, to select transconjugant cells (which result from the transfer of plasmids, via conjugation, between ampicillin resistant (donors) and sensitive (recipient) strains).

3.3.4 Co-cultures in Structured Media

We inoculated the sensitive and ampicillin-resistant strains of each demographic combination, on petri-dishes containing solid LB medium (supplemented with 1.5 % agar) and incubated at 37ºC for 24 hours.

After the incubation period, the whole resulting bacterial culture was retrieved and suspended on 2 ml solution of $MgSO_4$ (0.01 M). Samples of the co-cultures were then retrieved, appropriately diluted and plated on selective media, as described for the unstructured media.

3.3.5 Data analysis

We calculated the relative fitness (W_S) of the sensitive strain in each combination of conditions, both for ampicillin and ampicillin-free co-cultures, using the following equation (Lenski *et al*, 1991):

$$\omega_s = \frac{Log\left(S_{final} / S_{initial}\right)}{Log\left(R_{final} / R_{initial}\right)}$$

where S is the population of ampicillin-sensitive strain, and R the population of ampicillin resistant strain, calculated in CFU ml $^{-1}$, at the beginning and end of the experiments.

Since transconjugant cells also produce β-lactamase, those present in the final population were subtracted from the original sensitive population (whence they were obtained, having the same chromosomal marker) and added to the resistant population.

The relative fitness of sensitive cells in presence of ampicillin was normalized with the relative fitness of sensitive cells in absence of ampicillin:

$$rFS = \omega_s(\text{with amp}) / \omega_s(\text{without amp})$$

In this equation $\omega_s(\text{with amp})$ and $\omega_s(\text{without amp})$ stand for fitness of sensitive cells if ampicillin is present or absent, respectively. By dividing these two fitness values, we automatically discount the fitness effect of the *Δara Val^R* chromosomal marker in the producer cells.

The resulting value was designated rFS, which represents the ability or the inability of sensitive cells to survive and/or grow under the protection of β-lactamase producing strains in an ampicillin enriched environment. The calculation of this ratio allowed us to discount potential pre-existing interference detected in the relative fitness of sensitive cells resulting from the co-cultures without ampicillin. A negative value of rFS means that sensitive cells survived but their numbers diminished overtime. If bacterial replication is faster than bacterial death, rFS becomes positive, implying that the number of sensitive cells is higher at the end than initially.

To understand the impact of transconjugants, we also calculated a second quantity, rFSb, which assumes that transconjugants do not produce β-lactamase and, as such, remain sensitive to ampicillin. In other words, contrary to the method used to calculate rFS, there is no subtraction of transconjugants from the sensitive population nor their addition to the resistant population. Considering that transconjugants do not produce β-lactamase is a non-realistic assumption but a comparison between rFS and rFSb is useful to assess the impact of conjugation on the protection levels.

Statistical tests and graphical analysis were performed in IBM SPSS Statistics version 23 and R version 3.2.0, available at http://www.rstudio.com/ (R Core Team, 2015).

3.4 RESULTS

3.4.1 β-lactamase producers protect sensitive cells from ampicillin in most conditions

We started by confirming that, after being in the presence of the clinically relevant concentration of ampicillin (Foulds, 1986) for 24 hours, sensitive cells do not form colonies when plated in selective media and incubated for 48 hours. This result was obtained regardless of the initial conditions: three total initial cell densities (circa 10^7 cells ml^{-1}, 10^5 cells ml^{-1} and 10^3 cells ml^{-1}) and two habitat structures (in liquid medium with agitation and on a Petri dish). In the same conditions, and contrary to what happened with sensitive cells, β-lactamase producing cells (cells harbouring each of the three plasmids) are able to grow and form colonies.

We then tested the ability of different β-lactamase producing strains to protect the sensitive strain, in ampicillin-enriched media. To do so, the sensitive strain was co-cultured with β-lactamase producing strains in 54 different conditions.

In presence of ampicillin and when co-cultured with β-lactamase producing strains, sensitive cells can either be: i) Protected, if the environment is indeed detoxified – leading to the survival of some sensitive cells; or ii) Eliminated, if producers are unable to effectively detoxify the environment – letting all sensitive cells die from the action of ampicillin. When protection of sensitive cells was observed, we further distinguish two possible outcomes: i) Survival of sensitive cells – when, after incubation, the number of sensitive cells decreased, but not all cells disappear; or ii) Growth of sensitive cells – when the numbers of sensitive cells increased over the incubation period. We observed protection in 34 out of the 54 tested conditions, among which there were 13 cases of growth of sensitive cells and 21 cases of survival.

3.4.1.1 Number of protection cases increases with initial total cell density

Fig. S1A shows that the number of protection cases increases with initial total cell density (henceforth referred to as “Density”): two cases with the lower Density, 14 cases with intermediate Density, and all of the 18 cases when Density is high. The number of cases where we observed growth of sensitive cells also revealed positive density

dependence: two cases when Density is low, four cases for intermediate Density, and seven cases at high Density.

3.4.1.2 Structured habitat has more cases of protection

In structured habitat, we observed protection of sensitive cells in 20 out of 27 conditions (Fig. S1A or S1B) and growth of sensitive cells in 11 of those conditions. In the unstructured habitat, sensitive cells were protected in 14 out of 27 conditions (Fig. S1A or S1B) and only had a net increase in numbers in two of those.

3.4.1.3 Bacteria harbouring natural plasmids efficiently detoxify ampicillin-enriched environments

All three plasmids used in this work detoxify the environment in several conditions (Fig. S1A or S1B). Even for plasmid R1, the lowest protective plasmid of the three, we observed nine cases of protection (out of 18 conditions), which corresponds to 50% of the cases. Furthermore, we observed 13 protection cases (out of 18) with the RP4 plasmid (72%) and 12 protection cases (out of 18) with the R16a plasmid (67%). The difference between plasmids is even stronger if one looks to the cases of growth of sensitive cells: plasmid RP4 enabled the growth of sensitive cells in seven out of 18 cases (39%), whereas with plasmid R16a we observed only four cases (22%) and only two cases with R1 (11%).

3.4.1.4 Effect of initial cell frequency in the number of protection cases

Cases of protection increase with the initial frequency of sensitive cells (henceforth referred to as “Frequency”) but only slightly: we observed 10 protection cases with low Frequency, 11 cases at intermediate Frequency and 13 cases at the highest Frequency (Fig. S1B). A single trend is absent if one looks at the cases of growth of sensitive cells: four, three and six cases, when Frequency were high, intermediate and low, respectively.

3.4.2 An ANOVA shows that plasmids, the three demographic conditions, and all interactions, significantly affect the levels of protection

We quantified protection levels by measuring the fitness of sensitive cells in relation to producer cells, rFS (see Table S1). A four-way ANOVA was conducted to compare the effects of the conditions on protection. All the conditions as well as all their interactions significantly affect the levels of protection ($p<0.001$ for all cases, Table 1).

We also quantified the effect size of each factor using the partial η^2, an index that describes the extent of an effect after controlling for the impact of other variables in a model (Levine and Hullett, 2002), and found that all factors and their interactions have high or very high effect sizes.

Table 1. Four-way ANOVA with p-values and effect size (partial η^2) for each factor and for each interaction between factors.

Variable	df	F	p-value	partial η^2
Plasmid	2	546.9	< 0.001	0.910
Structure	1	1189.1	< 0.001	0.917
Density	2	1852.0	< 0.001	0.972
Frequency	2	81.2	< 0.001	0.601
Plasmid x Structure	2	56.2	< 0.001	0.510
Plasmid x Density	4	71.4	< 0.001	0.726
Plasmid x Frequency	4	22.4	< 0.001	0.454
Structure x Density	2	53.8	< 0.001	0.499
Structure x Frequency	2	30.4	< 0.001	0.360
Density x Frequency	4	318.1	< 0.001	0.922
Plasmid x Structure x Density	4	115.9	< 0.001	0.811
Plasmid x Structure x Frequency	4	73.6	< 0.001	0.732
Plasmid x Density x Frequency	8	116.1	< 0.001	0.896
Structure x Density x Frequency	4	120.6	< 0.001	0.817
Plasmid x Structure x Density x Frequency	8	47.4	< 0.001	0.778

3.4.2.1 Effect of initial total cell density on the level of protection

Initial total cell density (Density), is the condition with stronger effect size on the protection of sensitive cells (rFS) ($F(2,108) = 1851.98$, $p < 0.001$, partial $\eta^2 = 0.972$). *Post hoc* comparisons using the Tukey HSD test (Fig. 1) indicated that the mean score (M) for high Density (M = -0.229, SD = 0.013) is significantly higher than that of intermediate Density (M = -0.511, SD = 0.013). Additionally, lower Density is where the lowest mean

score of rFS is observed (M = -1.305, SD = 0.013). Overall, these results reveal that rFS increases with Density.

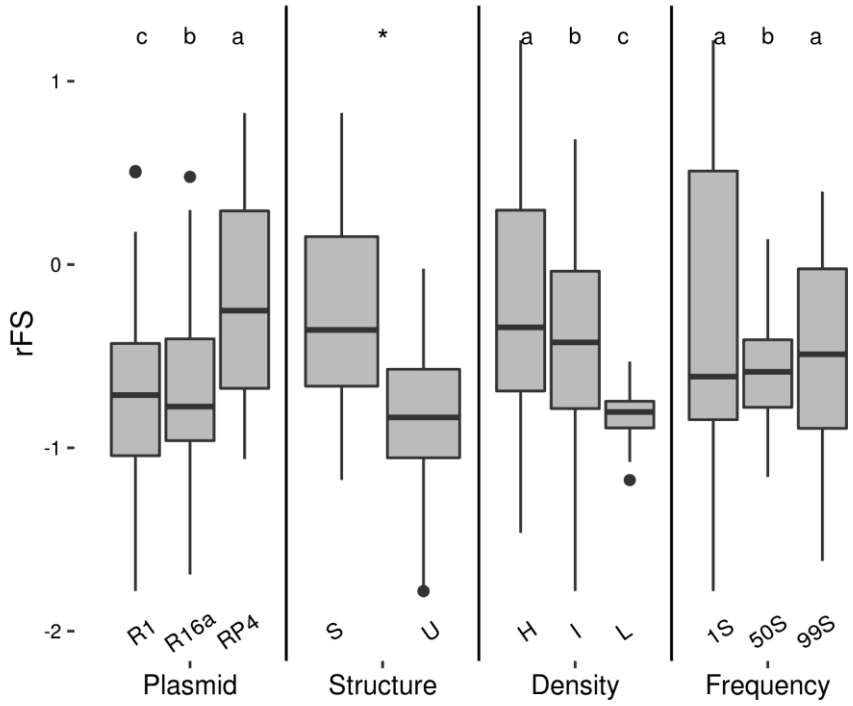

Figure 1. Level of Protection of the sensitive bacteria (rFS) for each subgroup of conditions. rFS values not sharing the same letter are significantly different (Tukey HSD, $p < 0.05$). * rFS values significantly different (see Table 1). "R1", "R16a" and RP4" stand for E. coli cells harboring plasmids R1, R16a or RP4 respectively. "S" stands for structured and "U" for unstructured habitats. "H", "I" and "L" stand for high, intermediate and low initial total density. "1S", "50S" and "99S" stand for the initial strain frequencies 1S:99R, 50S:50R, and 99S:1R, respectively, where "S" stands for sensitive and "R" stands for resistant (producers of β-lactamase). Outlier values are not represented. For the categories "Plasmid", "Density" and "Frequency" n=18 for each group, while for the category "Structure" n = 27.

3.4.2.2 Effect of habitat structure on the level of protection

Habitat structure also significantly affect the protection levels ($F(1,108) = 1189.07$, $p < 0.001$, partial $\eta^2 = 0.917$). The observed mean scores of rFS, for each structure, showed higher levels of protection on structured habitat (M = -0.423, SD = 0.011), than in the unstructured (M = -0.940, SD = 0.011) (Fig. 1).

3.4.2.3 Effect of plasmids on the level of protection

The plasmid harboured by the β-lactamase producing cells also has a significant effect on the levels of protection ($F(2,108) = 546.88$, $p < 0.001$, partial $\eta^2 = 0.910$). We further conducted a *post hoc* comparison using the Tukey HSD test, to understand the differences of protection between plasmids. We observed that the mean score (M) of rFS for plasmid RP4 (M = -0.368, SD = 0.013) is significantly higher than that the other two plasmids. Furthermore, the mean score for the R16a plasmid (M = -0.703, SD = 0.013) is significantly higher than that of the R1 plasmid (M = -0.974, SD = 0.013).

3.4.2.4 Effect of initial frequency on the level of protection

The initial frequency of sensitive cells (Frequency), also significantly affects the levels of protection. This demographic factor also has a strong effect size on the protection of sensitive cells, but not so strong as the other isolated conditions ($F(2,108) = 81.22$, $p < 0.001$, partial $\eta^2 = 0.601$). The effect size resulting from the interaction between Density and Frequency was more pronounced ($F(4,108) = 318.10$, $p = < 0.001$, partial $\eta^2 = 0.922$), being superior to that of the structure, plasmid or Frequency alone or other interactions (e.g. the interaction "Structure x Density" has partial $\eta^2 = 0.499$ only). For example, in the case involving producers of β-lactamase harbouring the R1 plasmid, at structured habitat, Fig. 2 illustrates the effect of the interaction between Density and Frequency: the line obtained at high Density is not parallel to the lines obtained at low and intermediate Densities. In other words, for this case, protection levels are higher at higher Densities, but even higher if Frequency is intermediate or low (Fig. 2).

Additionally, a post hoc Tukey HSD test was used to assess the differences between the three studied initial frequencies of sensitive cells revealed that the intermediate Frequency (50S:50R) has a mean score significantly lower than the other two frequencies (M = -0.816, SD = 0.013) (Fig. 1).

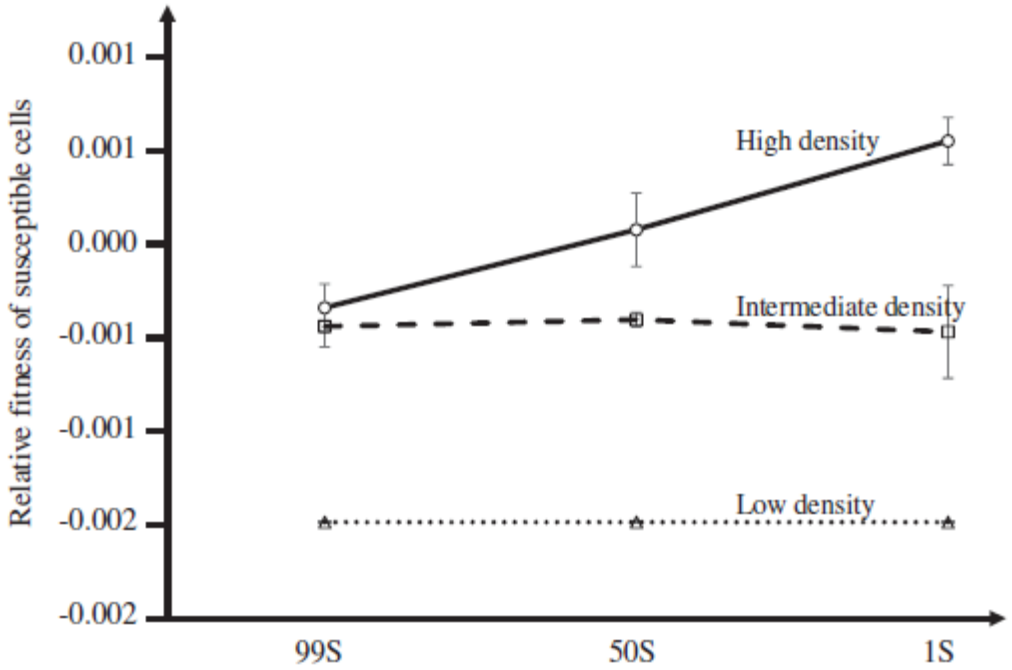

Figure 2. Interaction plot of fitted relative fitness of susceptible cells (rFS), showing how the protection levels depends on Frequency and on Density. For this specific case (involving producers of β-lactamase harbouring the R1 plasmid, at structured habitat), one can see that protection levels are higher at higher Densities, but even higher if Frequency is intermediate (50S:50R) or low (1S:99R). Error bars show twice the standard error around the means.

3.4.3 Initial total density and structure are demographic predictors of protection

To measure the weight of each condition on the level of protection (rFS), we also performed a forward stepwise regression analysis of the effects of these conditions on the level of protection (Table 2). Results were in agreement with the conclusions withdrawn from the ANOVA analysis. The factor Density alone is the demographic condition that has a more pronounced effect on rFS values. Indeed, Density alone explains 26% of the observed protection of sensitive cells (R^2 = 0.260, p < 0.001), followed by structure (+18%, R^2 = 0.440, p < 0.001), and the presence of RP4 plasmid in the β-lactamase producer cells (+5.9%, R^2 = 0.499, p < 0.001).

These results show that about 50% of the variance of the protection (rFS) values are explained by these three conditions: Density, Habitat Structure and harbouring the RP4 plasmid.

The remaining conditions, such as Frequency or harbouring the R16a or R1 plasmid, have no further significant contribution to the stepwise regression.

Table 2. Stepwise regression for rFS values.

Variable	Individual R^2	Cumulative R^2	Standard Error
Density	0.260	0.260	0.669
Structure	0.180	0.440	0.582
RP4 Plasmid	0.059	0.499	0.551

3.4.4 Plasmid transfer does not influence the impact of demographic conditions on the levels of protection

β-lactamase genes of producer cells were all encoded on plasmids that are able to transfer to ampicillin-sensitive cells, during the co-culture experiments, originating transconjugant cells (Table S2). Transconjugant cells are also β-lactamase producers, hence rFS values were always calculated assuming that transconjugant cells are also producers (see Materials and Methods).

Therefore, we compared rFS values with a second set of values (rFSb) assuming that transconjugants do not produce β-lactamase and as such remain sensitive to ampicillin. Despite being a non-realistic assumption, rFSb was calculated to assess the impact of conjugation on the protection levels. In ten cases the rFSb values where significantly higher than rFS values (Table S1). Despite this difference, the ANOVA using rFSb values yielded the same qualitative conclusions as with rFS (Table S3). Furthermore, the conditions where there is protection are the same.

3.5 DISCUSSION

With this work we had two main objectives. First, to confirm that there is protection of sensitive cells by producers of β-lactamase. Since the answer could depend on the plasmid, we use three plasmids of natural origin, and because the answer could also depend on specific conditions, we checked different demographic conditions of habitat structure, initial strain frequency and initial total cell density, in a total of 18 combinations for each of the plasmid. Contrary to what was done in previous studies, here, we used initial strain frequencies (Frequency) and total initial cell densities (Density) spanning four orders of magnitude each. We used this broader range of initial cell frequencies and densities since it was important to include most contrasting scenarios. Given that we tested all combinations of these conditions we could also achieve our second goal: to understand how these demographic conditions interact and affect the levels of protection.

We found that, in 34 of the 54 studied conditions, bacteria expressing β-lactamase protect ampicillin-sensitive cells from otherwise lethal doses of ampicillin. The best condition for β-lactamase producers to protect sensitive cells is the combination of the highest Density when initial sensitive cells are rare (low Frequency). In 13 conditions, the population of sensitive bacteria was even able to increase in numbers.

Although there are more cases of protection and growth of sensitive cells in structured habitat (20 cases), it is interesting that so many cases of protection were observed in unstructured environment (14 cases). Since in liquid media, the effect of β-lactamase is diluted across the whole environment (and not localized around a producer cell as in structured environment), this indicates that producers are highly effective at detoxifying the environment.

In most cases, the relative fitness of sensitive cells increases with initial total cell density, as predicted by (Ross-Gillespie *et al*, 2009). The effect of initial strain frequency, however, is not so easy to predict. As pointed out by (Ross-Gillespie et al, 2007) the fitness of cheaters (here sensitive cells) should not depend on cheaters' frequency in the population if selection is weak (Hamilton, 1964). However, with some degree of

population structuring or in cases in which higher frequency of cooperators would lead to greater population growth, the fitness of sensitive cells should depend on strain frequency (Ross-Gillespie *et al*, 2007).

Our results show that relative fitness of sensitive cells indeed depends on the initial strain frequency. However, the effect of Frequency is markedly dependent on other conditions, mainly initial total cell density, as shown by the very low p-values of their interactions (Table 1). This is clearer by looking at effect sizes (Table 1, partial η^2 values): the effect size of the interaction "Frequency x Density" is the second highest value appearing in Table 1 (partial $\eta^2 = 0.922$), whereas that of "Frequency" condition isolated is partial $\eta 2 = 0.601$, one of the lowest values appearing in Table 1. For example, in structured habitat and using the RP4 or R16a plasmids, the level of protection is higher when sensitive cells are rare with high Density, whereas at low Density, the level of protection is higher when the initial proportion of sensitive cells is higher (Fig. S1B).

E. coli's main habitats are believed to be the lower intestine of warm-blooded animals (Savageau, 1983). Given that these habitats are structured, probably most *E. coli* cells close to a given *E. coli* cell belong to the same "colony", that is, probably most of them harbour the β-lactamase gene. Protection may be useful in this case if a certain proportion of kin cells, despite coding for β-lactamase, is transiently not producing the enzyme – the so-called phenotypic heterogeneity (Ackermann, 2015). Therefore, by protecting neighbouring cells, producers of β-lactamase are protecting their kin, thus maximizing their inclusive fitness (Hamilton, 1964). Moreover, given that cells are in a structured environment, there is accumulation of β-lactamase near producer cells, hence allowing for higher (local) concentration of the enzyme.

However, other (different) bacterial strains of *E. coli* may be present in the same habitat, e.g. mammalian gut (Caugant *et al*, 1981; Faith *et al*, 2013), so producers of β-lactamase may be protecting other strains as well. In this case, one should expect β-lactamase producer *E. coli* cells to be more refractory to exploitation by other cells not producing public-goods. At least in many other systems, mechanisms to avoid exploitation by cheaters have evolved (West *et al*, 2006).

The theoretical prediction is that selection for such mechanisms is expected to occur when relatedness is low (if relatedness is high, control mechanisms are not necessary) (Dionisio and Gordo, 2007; Frank, 1995). This theoretical prediction, together with our observations that there is protection in so many conditions (hence not avoiding helping other cells), is an indication that, even if different *E. coli* cells co-inhabit, the two populations are not mingled. Therefore, within the surrounding of each *E. coli* cell, genetic variance is much lower than between patches, which implicates that relatedness is high (Frank, 1998).

Mammalian hosts harbour many bacterial species (Faith *et al*, 2013; Martinez *et al*, 2013; Qin *et al*, 2010), implying that some of the protected bacterial cells may belong to other species. The ability of *E. coli* to protect sensitive bacterial cells from other species has already been experimentally demonstrated (Perlin *et al*, 2009). This may be advantageous to transferable plasmids. Indeed, since they are able to transfer to many bacterial species (Dionisio *et al*, 2002), protected cells are, potentially, new hosts for the plasmid. From this point of view, the production of high amounts of β-lactamase to save bacteria not carrying the plasmid become advantageous to the plasmid itself.

An important point of this work was the use of plasmids of natural origin, contrary to previous works where protection was also observed (Clark *et al*, 2009; Dugatkin *et al*, 2005; Perlin *et al*, 2009; Yurtsev *et al*, 2013). None of the three plasmids was engineered to increase or optimize for β-lactamase production. In fact, all three plasmids used in this work (R1, R16 and RP4) were obtained from natural isolates. RP4 was isolated from *Pseudomonas aeruginosa* (Datta *et al*, 1971), R1 from *Salmonella enterica* serovar paratyphi (Anderson and Datta, 1965) and R16a from *Providencia stuartii* (Chabbert *et al*, 1972). The three plasmids are low copy-number (Grinsted *et al*, 1972; Szabo *et al*, 2016; Uhlin and Nordstrom, 1975) and encode TEM β-lactamases (class A) (Matthew and Hedges, 1976; Szabo *et al*, 2016), so the three enzymes are very similar. It has been reported that the level of transcription of RP4 β-lactamase is higher than that of R1 plasmid (Crowlesmith and Howe, 1980), which explains the higher levels of protection displayed by RP4 in our results and the predominance of RP4 in our stepwise analysis (Table 2).

It could be argued that a weakness of our study is that we used a domesticated bacterial strain to house the plasmids, rather than wild-type isolates. However, our strain was not specifically engineered to increase or optimize β-lactamase production. Moreover, the fact that we used a domesticated strain revealed serendipitous because it happened to be the same *E. coli* strain used in the study where protection was not observed (Medaney *et al*, 2016).

The interaction between demographic conditions explored in this study explains why previous works did not agree on the existence of protection. Since contrasting results are obtained with different demographic conditions, it is possible that some previous works explored propitious conditions (hence observing protection of sensitive cells), while others did not.

Our results also suggests an explanation for how is it possible that ampicillin-sensitive bacteria are isolated from subjects undergoing ampicillin treatment (Brook, 2004). Despite the presence of the antibiotic in these patients, probably some sensitive cells never encountered it, due to the β-lactamase produced by neighbouring resistant cells.

The experiments described here were performed a laboratorial environment; in the future, however, this should be tested in a natural environment (e.g. in mice gut, as recently done with the antibiotic chloramphenicol (Sorg *et al*, 2016)).

DATA ARCHIVING

All data are available as Supplementary Information files.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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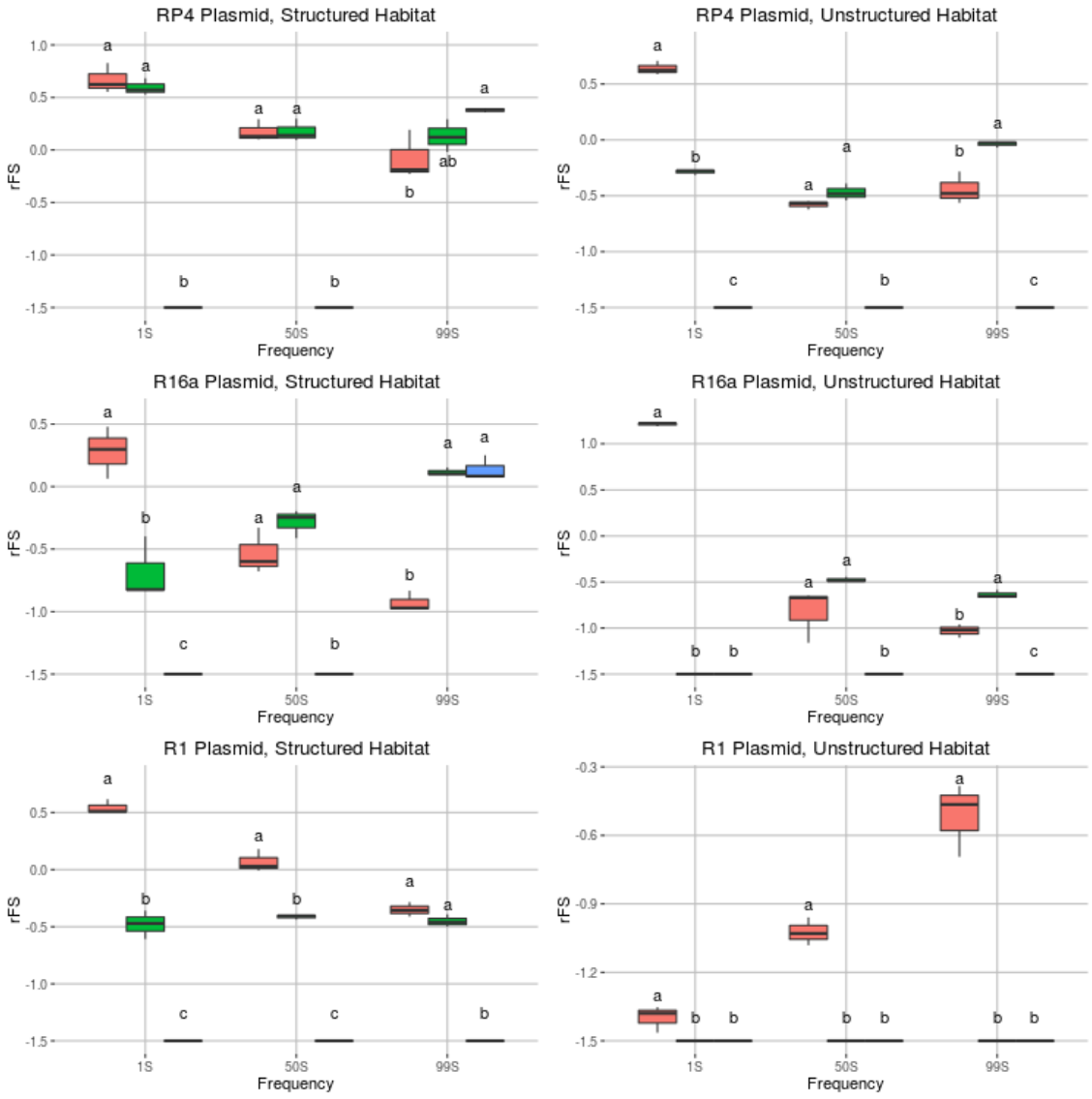
3.7 SUPPLEMENTARY INFORMATION

A



B

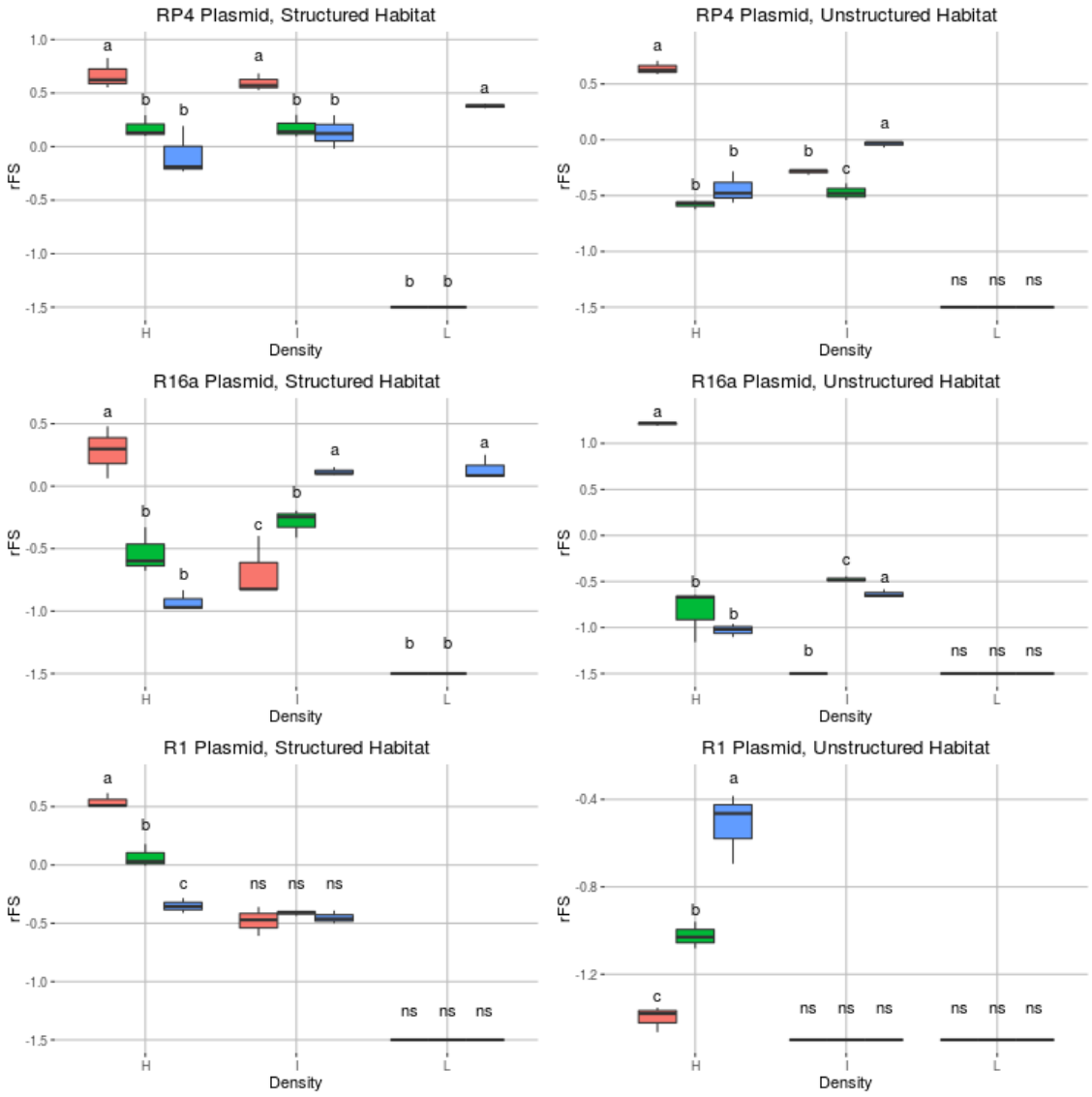


Figure S1. Level of Protection of the sensitive bacteria (relative frequency of sensitive cells, rFS) by *E. coli* cells harbouring each plasmid R1, R16a or RP4 for each type of habitat (habitat structure). **A:** rFS values are represented for each "Frequency": as in Fig.1, "1S" stands for 1S:99R, "50S" for 50S:50R, and "99S" for 99S:1R, where "S" stands for sensitive cells and "R" stands for resistant cells (producers of β-lactamase). Within each "Frequency", rFS values are separated according to each of the three "Densities": Red for High "Density", green for Intermediate "Density" and blue for Low "Density". **B:** rFS values are represented for each Density: as Fig. 1, "H stands for High Density, I for Intermediate, and L for Low Density. Within each of Density, rFS values are separated according to each of the three Frequencies: red for 1S:99R, green for 50S:50R and blue for 99S:1R. rFS values not sharing the same letter are significantly different (Tukey HSD, $p < 0.05$).

Table S1. Fitness without and with Ampicillin, rFS and rFSb values for each condition. Values presented are the mean score for the fitness of sensitive cells without and with ampicillin, rFS and rFSb. The values for each condition always results from three replicas. 2xStdErr stands for twice the standard error. Student T-test to compare rFS and rFSb values is also presented (T_test). For each condition: R1, R16a and RP4 stand for the plasmids coding for β-lactamase. As in Fig.1, "S" stands for structured and "U" for unstructured habitats. Likewise, "H" stands for High Density, "I" for Intermediate Density, "L" for Low Density, "1S" stands for Frequency 1S:99R, "50S" for Frequency 50S:50R, and "99S" for Frequency 99S:1R.

Conditions	rFS	2xStdErr	rFSb	2xStdErr	T_test	sig
RP4:S:H:99S	-0.075	0.466	0.324	0.128	0.088	ns
RP4:S:H:50S	0.174	0.208	0.412	0.023	0.056	ns
RP4:S:H:1S	0.668	0.285	0.905	0.074	0.094	ns
RP4:S:I:99S	0.132	0.312	0.315	0.149	0.169	ns
RP4:S:I:50S	0.177	0.213	0.377	0.127	0.061	ns
RP4:S:I:1S	0.594	0.160	0.660	0.127	0.333	ns
RP4:S:L:99S	0.379	0.041	0.379	0.041	1.000	ns
RP4:S:L:50S	-0.759	0.031	-0.759	0.031	1.000	ns
RP4:S:L:1S	-0.684	0.046	-0.684	0.046	1.000	ns
RP4:U:H:99S	-0.442	0.287	-0.012	0.082	0.028	*
RP4:U:H:50S	-0.579	0.077	-0.190	0.120	0.001	**
RP4:U:H:1S	0.639	0.124	0.950	0.056	0.005	**
RP4:U:I:99S	-0.039	0.053	0.104	0.047	0.002	**
RP4:U:I:50S	-0.471	0.153	-0.052	0.147	0.002	**
RP4:U:I:1S	-0.287	0.047	0.167	0.051	0.000	***
RP4:U:L:99S	-0.868	0.034	-0.868	0.034	1.000	ns
RP4:U:L:50S	-1.017	0.106	-1.017	0.106	1.000	ns
RP4:U:L:1S	-0.820	0.047	-0.820	0.047	1.000	ns
R16a:S:H:99S	-0.928	0.166	-0.451	0.046	0.007	**
R16a:S:H:50S	-0.535	0.365	-0.166	0.199	0.052	ns
R16a:S:H:1S	0.280	0.417	0.450	0.140	0.290	ns
R16a:S:I:99S	0.116	0.064	0.120	0.060	0.875	ns
R16a:S:I:50S	-0.285	0.226	-0.163	0.170	0.217	ns
R16a:S:I:1S	-0.683	0.492	-0.338	0.022	0.136	ns
R16a:S:L:99S	0.138	0.196	0.138	0.196	1.000	ns
R16a:S:L:50S	-0.819	0.020	-0.819	0.020	1.000	ns
R16a:S:L:1S	-1.085	0.173	-1.085	0.173	1.000	ns
R16a:U:H:99S	-1.028	0.146	-0.574	0.139	0.001	**
R16a:U:H:50S	-0.826	0.578	-0.510	0.088	0.196	ns
R16a:U:H:1S	1.212	0.037	1.280	0.071	0.060	ns
R16a:U:I:99S	-0.636	0.091	-0.591	0.041	0.220	ns
R16a:U:I:50S	-0.474	0.051	-0.474	0.051	1.000	ns
R16a:U:I:1S	-1.664	0.083	-1.664	0.083	1.000	ns
R16a:U:L:99S	-0.968	0.029	-0.968	0.029	1.000	ns
R16a:U:L:50S	-0.784	0.018	-0.784	0.018	1.000	ns
R16a:U:L:1S	-0.792	0.087	-0.792	0.087	1.000	ns

R1:S:H:99S	-0.351	0.128	-0.351	0.128	0.996	ns
R1:S:H:50S	0.067	0.198	0.069	0.198	0.983	ns
R1:S:H:1S	0.543	0.127	0.545	0.127	0.968	ns
R1:S:I:99S	-0.451	0.110	-0.451	0.110	1.000	ns
R1:S:I:50S	-0.415	0.038	-0.415	0.038	1.000	ns
R1:S:I:1S	-0.480	0.249	-0.480	0.249	1.000	ns
R1:S:L:99S	-0.741	0.035	-0.741	0.035	1.000	ns
R1:S:L:50S	-0.545	0.048	-0.545	0.048	1.000	ns
R1:S:L:1S	-0.616	0.047	-0.616	0.047	1.000	ns
R1:U:H:99S	-0.514	0.322	-0.370	0.065	0.257	ns
R1:U:H:50S	-1.024	0.122	-0.440	0.112	0.000	***
R1:U:H:1S	-1.399	0.117	-0.551	0.098	0.000	***
R1:U:I:99S	-1.595	0.040	-1.595	0.040	1.000	ns
R1:U:I:50S	-1.520	0.051	-1.520	0.051	1.000	ns
R1:U:I:1S	-1.756	0.046	-1.756	0.046	1.000	ns
R1:U:L:99S	-0.922	0.050	-0.922	0.050	1.000	ns
R1:U:L:50S	-0.770	0.020	-0.770	0.020	1.000	ns
R1:U:L:1S	-2.047	4.113	-2.047	4.113	1.000	ns

Table S2. Final number of transconjugant cells. For each of the conditions, the table displays the mean score for: the final number of transconjugant cells (in cfu/ml), for experiments both with and without Ampicillin; and the proportion of cells that received the plasmid in the final of each experiment, both with and without Ampicillin. The values for each condition always results from three replicas. 2xStdErr stands for twice the standard error. For each condition: R1, R16a and RP4 stand for the plasmids coding for β-lactamase. As in Fig.1, "S" stands for structured and "U" for unstructured habitats. Likewise, "H" stands for High Density, "I" for Intermediate Density, "L" for Low Density, "1S" stands for Frequency 1S:99R, "50S" for Frequency 50S:50R, and "99S" for Frequency 99S:1R.

Conditions	T (cfu/ml) w/out Ampicillin	2xStdErr	T (cfu/ml) with Ampicillin	2xStdErr	ratio T/S w/out Ampicillin	2xStdErr	ratio T/S with Ampicillin	2xStdErr
RP4:S:H:99S	9.77E+09	7.15E+09	8.21E+08	8.75E+08	0.237	0.033	1.252	1.138
RP4:S:H:50S	1.70E+10	7.05E+09	2.25E+09	1.50E+09	0.726	0.320	0.997	0.270
RP4:S:H:1S	4.53E+07	6.21E+07	2.73E+07	2.41E+07	0.089	0.094	0.625	0.422
RP4:S:I:99S	7.60E+08	1.42E+09	2.24E+07	2.08E+07	0.083	0.117	0.844	0.449
RP4:S:I:50S	3.08E+09	1.96E+09	7.61E+06	1.47E+07	0.215	0.112	0.885	0.313
RP4:S:I:1S	3.33E+07	4.16E+07	1.20E+06	2.77E+06	0.438	0.518	0.340	0.759
RP4:S:L:99S	3.04E+07	1.34E+07	0.00E+00	0.00E+00	0.001	0.000	0.000	0.000
RP4:S:L:50S	1.13E+08	1.31E+08	0.00E+00	0.00E+00	0.010	0.007	0.000	0.000
RP4:S:L:1S	3.41E+07	2.00E+07	0.00E+00	0.00E+00	0.009	0.007	0.000	0.000
RP4:U:H:99S	5.33E+08	1.17E+09	1.11E+08	3.01E+07	0.039	0.069	1.171	0.474
RP4:U:H:50S	9.87E+08	1.77E+09	1.30E+07	8.00E+06	0.182	0.226	0.904	0.078
RP4:U:H:1S	2.60E+07	3.42E+07	7.53E+07	2.34E+07	0.250	0.322	0.773	0.114
RP4:U:I:99S	3.67E+08	6.11E+08	4.46E+06	2.53E+06	0.029	0.050	0.870	0.024
RP4:U:I:50S	3.77E+09	2.31E+08	3.33E+05	5.67E+05	0.646	0.569	1.045	0.079
RP4:U:I:1S	9.00E+07	5.29E+07	6.27E+04	2.67E+04	0.312	0.202	1.122	0.337
RP4:U:L:99S	7.33E+08	9.45E+08	0.00E+00	0.00E+00	0.046	0.067	0.000	0.000
RP4:U:L:50S	4.03E+09	2.86E+09	0.00E+00	0.00E+00	0.305	0.172	0.000	0.000

RP4:U:L:1S	1.03E+08	1.30E+08	0.00E+00	0.00E+00	0.400	0.599	0.000	0.000
R16a:S:H:99S	4.96E+09	1.70E+10	1.62E+06	4.92E+05	0.244	0.834	1.028	0.175
R16a:S:H:50S	3.66E+09	1.24E+10	1.05E+06	1.46E+06	0.219	0.722	0.941	0.095
R16a:S:H:1S	8.33E+06	1.34E+07	1.60E+06	1.39E+06	0.120	0.213	0.424	0.664
R16a:S:I:99S	3.35E+08	4.55E+08	5.67E+04	8.89E+04	0.017	0.034	0.067	0.113
R16a:S:I:50S	4.67E+08	4.74E+08	3.39E+03	1.09E+04	0.058	0.039	0.438	0.983
R16a:S:I:1S	1.01E+07	1.59E+06	1.87E+02	1.51E+02	0.042	0.020	0.833	0.577
R16a:S:L:99S	6.37E+07	2.17E+08	0.00E+00	0.00E+00	0.001	0.003	0.000	0.000
R16a:S:L:50S	9.29E+05	4.45E+05	0.00E+00	0.00E+00	0.000	0.000	0.000	0.000
R16a:S:L:1S	5.68E+05	7.49E+05	0.00E+00	0.00E+00	0.003	0.004	0.000	0.000
R16a:U:H:99S	3.83E+07	5.88E+07	5.50E+05	1.40E+05	0.002	0.002	1.567	1.906
R16a:U:H:50S	1.38E+09	9.45E+08	1.14E+05	5.97E+04	0.107	0.127	0.938	0.733
R16a:U:H:1S	1.32E+08	5.70E+07	9.77E+07	3.65E+07	0.774	0.078	0.256	0.098
R16a:U:I:99S	4.97E+07	6.21E+07	6.67E+02	7.57E+02	0.021	0.033	0.378	0.389
R16a:U:I:50S	3.00E+09	1.43E+09	0.00E+00	0.00E+00	0.214	0.278	0.000	0.000
R16a:U:I:1S	8.60E+07	5.55E+07	0.00E+00	0.00E+00	0.330	0.340	0.000	0.000
R16a:U:L:99S	1.06E+08	1.03E+08	0.00E+00	0.00E+00	0.015	0.011	0.000	0.000
R16a:U:L:50S	2.92E+09	2.10E+09	0.00E+00	0.00E+00	0.142	0.104	0.000	0.000
R16a:U:L:1S	9.23E+07	3.53E+07	0.00E+00	0.00E+00	0.326	0.284	0.000	0.000
R1:S:H:99S	4.14E+06	1.63E+06	1.03E+02	1.15E+01	0.000	0.000	0.004	0.004
R1:S:H:50S	1.96E+07	1.47E+07	1.60E+04	2.35E+04	0.000	0.000	0.017	0.032
R1:S:H:1S	3.15E+06	1.27E+06	1.39E+05	1.71E+05	0.003	0.000	0.011	0.011
R1:S:I:99S	5.65E+04	4.98E+04	0.00E+00	0.00E+00	0.000	0.000	0.000	0.000
R1:S:I:50S	3.28E+05	1.27E+05	0.00E+00	0.00E+00	0.000	0.000	0.000	0.000
R1:S:I:1S	1.50E+04	1.19E+04	0.00E+00	0.00E+00	0.000	0.000	0.000	0.000
R1:S:L:99S	1.35E+05	8.80E+04	0.00E+00	0.00E+00	0.000	0.000	0.000	0.000
R1:S:L:50S	2.61E+06	3.88E+06	0.00E+00	0.00E+00	0.000	0.000	0.000	0.000
R1:S:L:1S	1.98E+05	1.37E+05	0.00E+00	0.00E+00	0.000	0.000	0.000	0.000
R1:U:H:99S	1.35E+08	1.38E+08	2.59E+06	2.84E+06	0.004	0.002	0.618	0.568
R1:U:H:50S	4.00E+07	4.28E+07	2.10E+05	2.00E+04	0.003	0.003	1.241	0.466
R1:U:H:1S	8.22E+06	5.62E+06	6.81E+04	2.62E+04	0.040	0.030	1.211	0.382
R1:U:I:99S	1.30E+07	6.00E+06	0.00E+00	0.00E+00	0.001	0.001	0.000	0.000
R1:U:I:50S	2.87E+07	3.16E+07	0.00E+00	0.00E+00	0.002	0.003	0.000	0.000
R1:U:I:1S	7.47E+06	1.09E+06	0.00E+00	0.00E+00	0.029	0.012	0.000	0.000
R1:U:L:99S	1.60E+07	4.10E+06	0.00E+00	0.00E+00	0.001	0.000	0.000	0.000
R1:U:L:50S	2.33E+07	1.63E+07	0.00E+00	0.00E+00	0.002	0.002	0.000	0.000
R1:U:L:1S	8.39E+06	6.11E+05	0.00E+00	0.00E+00	0.036	0.016	0.000	0.000

Table S3. Four-way ANOVA with p-values and effect size (partial η^2) for each factor and for each interaction between factors using values of rFSb.

TABLE S3. **ANOVA and size effect of each factor using** rFSb **values**				
Variable	**df**	**F**	**p-value**	**partial η^2**
Plasmid	2	3131.54	< 0.001	0.983
Structure	1	3751.22	< 0.001	0.972
Density	2	12123.80	< 0.001	0.996
Frequency	2	296.52	< 0.001	0.846
Plasmid x Structure	2	34.48	< 0.001	0.390
Plasmid x Density	4	515.98	< 0.001	0.950
Plasmid x Frequency	4	102.83	< 0.001	0.792
Structure x Density	2	371.70	< 0.001	0.873
Structure x Frequency	2	213.44	< 0.001	0.798
Density x Frequency	4	1211.71	< 0.001	0.978
Plasmid x Structure x Density	4	335.32	< 0.001	0.925
Plasmid x Structure x Frequency	4	195.39	< 0.001	0.879
Plasmid x Density x Frequency	8	285.77	< 0.001	0.955
Structure x Density x Frequency	4	437.54	< 0.001	0.942
Plasmid x Structure x Density x Frequency	8	144.95	< 0.001	0.920

CHAPTER IV:

4 HARMING BEHAVIOR MEDIATED BY PLASMIDS

Manuscript in preparation

Iolanda Domingues, João Gama, Francisco Dionísio

4.1 ABSTRACT

Plasmids are known for decades and they seem to be ubiquitous in the bacterial world, but it is still unclear which forces maintain them among bacterial populations. Some plasmids carry helpful genes, but it is unclear why these genes are maintained in the plasmid and not recruited by the chromosome. Possibly, plasmids are maintained as parasites, however, plasmid transfer rates between bacteria seems to be too low. Here we show that bacteria use plasmids to harm plasmid-free cells, being able to slow down their growth. We performed growth experiments in a liquid environment while taking advantage of membranes with 0.4 μm pores. Using this pore size we block direct contact and bacterial conjugation between cells placed in the two opposite sides of the membrane, but allow the exchange of culture media and free metabolites between the two sides. We show that, in many cases, the growth rate of plasmid-free *Escherichia coli* cells is lower when mixed with *E. coli* cells harbouring a plasmid of natural origin (RP4, R16a or R1) than when the two populations are separated by the membrane. This was observed under six initial ecological conditions (density and frequency of cells with and without plasmid), and concluded that plasmids may be used as harming agents even in some cases where plasmid transfer was almost absent. To our knowledge, this is the first report demonstrating that conjugative plasmids may be used as biological weapons.

4.2 INTRODUCTION

In addition to the chromosome, bacterial cells often harbour plasmids. These double-stranded DNA molecules were discovered more than 60 years ago, but the reason for their existence is still a mystery.

Plasmids are by definition non-essential to bacterial cells, yet they frequently carry genes that are useful to their host – for instance, genes for antibiotic resistance, pathogen virulence or allelopathy. For that reason, it has been argued that plasmids can remain in bacterial populations due to their mutualistic nature. Carriage of useful genes cannot be a satisfactory explanation for the existence and maintenance of plasmids, since it raises another question: why does the cell keep the whole plasmid, instead of recruiting the useful genes into the chromosome and discarding the rest of the useless genes? Alternatively, such elements can be seen as molecular parasites, because they often inflict a fitness cost to their hosts. Additionally, some plasmids may even replicate and transfer to other bacterial cells – a process called bacterial conjugation, but that is mostly controlled by the plasmid itself, not by the bacterial cell. However, most plasmids seem to transfer in a very inefficient way, most often not compensating for the cost they inflict to their hosts (Stewart and Levin 1977). This is sometimes called the plasmid paradox or the Stewart – Levin paradox, after Frank Stewart and Bruce Levin (Stewart and Levin 1977, Gordon 1992, Bergstrom, Lipsitch et al. 2000).

A handful of other explanatory hypothesis have been proposed to solve this paradox (for a review, see (Dionisio, Nogueira et al. 2012)). A plasmid may be deleterious to its bacterial host upon its first arrival unto the cell; but after a period of co-evolution between plasmid and bacteria the burden can disappear (Bouma and Lenski 1988, Modi, Wilke et al. 1991, Dahlberg and Chao 2003, Dionisio, Conceicao et al. 2005, Harrison, Truman et al. 2015). Moreover, Bouma and Lenski (1988) observed that, in some cases, after adaptation bacterial cells may decrease their growth rate if they lose their plasmid. Additionally, plasmid-bacteria adaptation may have a side effect: when moving to another host, evolved conjugative plasmids occasionally confer a fitness advantage to it; this was observed even when the plasmids evolved in *Escherichia coli* and then moved to cells of another bacterial species— *Salmonella enterica* (Dionisio,

Conceicao et al. 2005). Plasmids are also implicated in the construction of bacterial biofilms (Ghigo 2001), structures that increase the resistance of bacterial cells to certain stresses, such as antibiotics. Another hypothesis comes from the observation that most strains are indeed bad donors of conjugative plasmids while only a small proportion of strains are excellent donors; perhaps these small proportion of excellent donors are responsible for the spread of conjugative plasmids among bacteria (Dionisio, Matic et al. 2002).Given their complexity, microbiomes would certainly contain some of these special strains.

A social hypothesis was, as well, proposed to explain the maintenance of plasmids. Some genes code for enzymes that display their function outside bacterial cells – as such, these enzymes constitute bacterial public goods. Therefore, by keeping these genes in transferable plasmids and allowing them to transfer to neighbouring cells (recipient cells that become transconjugants – cells or descendants of cells that just receive the plasmid), plasmid donors force transconjugants to pay the cost of carrying the plasmid as well as to produce the enzyme (Smith 2001, Nogueira, Rankin et al. 2009, Mc Ginty, Rankin et al. 2011, Rankin, Rocha et al. 2011, Nogueira, Touchon et al. 2012, Dimitriu, Lotton et al. 2014).

When formulating this hypothesis, Smith (2001) realized that the advantage of carrying public-good enzymes is twofold. First, by transferring the plasmid to other cells, these will also collaborate with the production of the public good – henceforth, we call this hypothesis “cooperation enforcement”. Second, plasmid donors impose the costs of harbouring the plasmid and of producing the public good to transconjugants – henceforth, we call this the “harming” hypothesis. This second advantage becomes even stronger because transconjugants will then be able to transmit the plasmid to other plasmid-free cells and so on. As such, any fitness cost payed by the original donors will largely be compensated by the cost imposed to the population of cells that just receive the plasmid (Dionisio, Nogueira et al. 2012). Already two decades ago, it has been shown that plasmids, upon entry into bacterial cells, sometimes induce a viable but nonculturable (VBNC) state in these cells – that is, transconjugants enter a state in which they are viable but lose the ability to grow on common culture media. To our knowledge, this has been observed only twice: (i) with a specific plasmid in *Pseudomonas fluorescens*

at specific temperatures (Oliver, Mcdougald et al. 1995); and (ii) with *E. coli* cells in biofilms (Hausner and Wuertz 1999).

In this work, we tested if plasmid-bearing cells can have an advantage only due to their harming effect: harming plasmid-free cells by transferring the plasmid to them (to which plasmid-free cells were still not adapted). For that, we had to disentangle the first and second advantages. Smith's initial hypothesis focused mostly on virulence factors. Some virulence factors are indeed excreted and have their main function outside bacterial cells – clearly acting as public goods. According to Smith's model, the fitness of each bacterial cell is higher if more cells produce the public good. Therefore, to focus on the harming effect, we studied resistance to ampicillin mediated by the production of β-lactamases coded in the plasmids of natural origin, RP4, R16a or R1. Each cell harbouring one of these plasmids produces a β-lactamase enzyme that is sufficient to locally detoxify the environment and save the producer from the effect of ampicillin. Because β-lactamase molecules destroy the antibiotic, they may be considered public-goods. However, contrary to the cases involving most or all virulence factors, the fitness of β-lactamase producers is not higher if more cells produce β-lactamase. In other words, if there is an advantage of bearing one of these plasmids it is just because of the second cause: donors force recipient cells to pay the cost of harbouring the plasmid and of producing β-lactamase.

In an attempt to test this hypothesis, we performed fitness measurements of plasmid-free bacterial cells when growing physically separated or together with plasmid-bearing cells, as well as the amount of newly formed transconjugants and their descendants. As expected, in many conditions conjugative plasmids lower the relative fitness of plasmid-free cells. However, it was highly unexpected to observe that the fitness of plasmid-free cells may decrease even if they did not receive the plasmid.

4.3 MATERIALS & METHODS

4.3.1 Bacterial Strains

In this work we used two isogenic strains of *Escherichia coli,* the wild-type *E. coli* K12 MG1655 and *E. coli* K12 MG1655 *Δara val*^{R}. The latter strain harbours two chromosomal markers: *Δara* – deletion of the arabinose operon which disables the metabolism of arabinose monosaccharide; and *val*^{R} – mutation that enables the growth in the presence of valine (40 μg L^{-1}) when isoleucine is absent.

Conjugative plasmids R1, R16a and RP4, of natural origin, were inserted into *E. coli* K12 MG1655 *Δara val*^{R}*,* through bacterial conjugation, resulting in the three ampicillin resistant strains: *E. coli* K12 MG1655 *Δara val*^{R} (R1), *E. coli* K12 MG1655 *Δara val*^{R} (R16a) and *E. coli* K12 MG1655 *Δara val*^{R} (RP4). All three β-lactamase producing strains are identical (except for the different plasmids) and will be referred to as plasmid-bearing or ampicillin-resistant strain. The wild-type strain will be referred to as plasmid-free or ampicillin-sensitive strain.

4.3.2 Ecological conditions of co-culture

The plasmid-free population was co-cultured with a population of plasmid-bearing cells, in a set of three different conditions: (i) each of the three different plasmid-bearing strains containing one of the three β-lactamase producing plasmids; (ii) two initial cell densities – High (10^{7} cells ml^{-1}) and Low (10^{5} cells ml^{-1}); (iii) three initial cell frequencies – 100:1, 1:1 and 1:100. The combination resulted in a total of 18 different combinations of conditions.

Each condition of co-culture was performed in parallel, when both cultures were physically mixed (Figures. 1A and 1B) or physically separated by the membrane (Figure. 1C and 1D).

Furthermore, each combination of conditions, without and with the membrane, was performed in ampicillin-free media (Figures. 1A and 1C) and in media containing ampicillin (100 μg ml^{-1}) (Figures. 1B and 1D). All co-cultures were performed in triplicate.

Additionally, and as a control, sensitive and resistant strains were cultured in isolation in media containing ampicillin (100 µg ml^{-1}) to confirm both the inability of the sensitive strain and the ability of the resistant strains to grow in the presence of the mentioned dosage of ampicillin.

4.3.3 Physically mixed co-cultures

Co-cultures of the two strains were performed in six-well plates. We inoculated the pair of strains, in each of the appropriate combinations, physically mixed in 5.7 mL of Luria broth (LB) and incubated at 37ºC with constant agitation, 130 rpm, (Figures. 1A and 1B). We allowed the bacteria to grow in co-culture for 24 hours (reaching the stationary phase).

After incubation, samples of the co-cultures were retrieved, appropriately diluted and plated on selective media: M9 Minimal Medium with 3 mM $MgSO_4$, supplemented with 4 g L^{-1} of arabinose to select cells with the marker of the sensitive strain; and supplemented with 4 g L^{-1} of glucose, 40 µg ml^{-1} of valine and 100 µg ml^{-1} of both ampicillin and kanamycin to select ampicillin resistant strains. Additionally, all samples were plated on M9 Minimal Media with 3 mM $MgSO_4$, supplemented with 4 g L^{-1} of arabinose, 40 µg ml^{-1} of valine and 100 µg/mL of both ampicillin and kanamycin, to select for transconjugant cells, resulting from the transfer of plasmids, via conjugation, between ampicillin resistant (donors) and sensitive (receptor) strains.

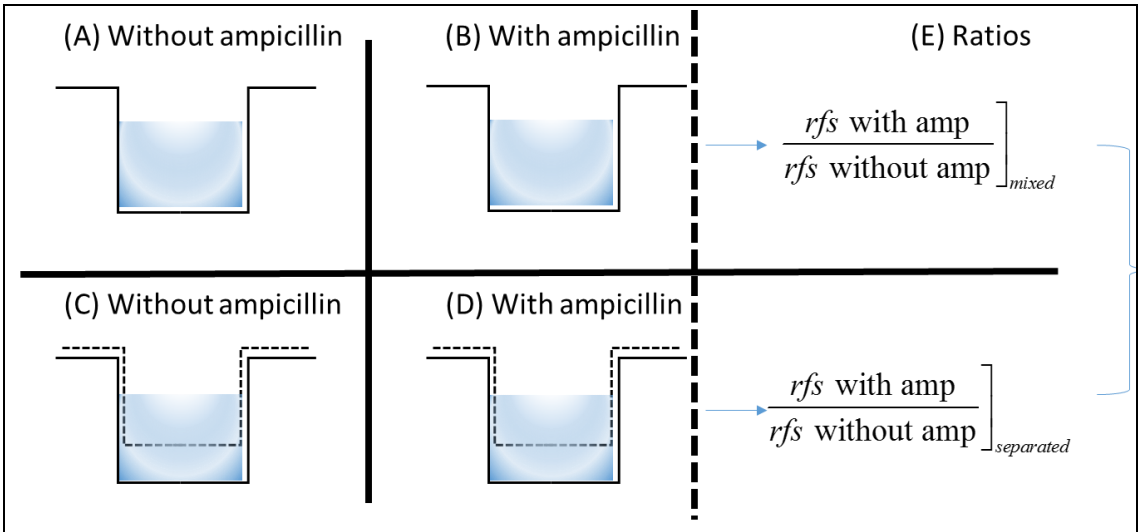


Figure 1. Competition experiments in 6-wells plates with or without membrane, and with or without ampicillin. Schematic outline of competitions where cells of the two populations can (A and B) or

cannot (C and D) establish physical contact, and where there is no antibiotic (A and C) or there is ampicillin (B and D). In (E) we show an outline of ratios calculated in the last sub-section of the Results section.

4.3.4 Physically separated co-cultures

Co-cultures of the two strains were also performed in six-well plates. This procedure was based on the method described in (Aoki, Pamma et al. 2005). We inoculated the pair of strains, in each of the appropriate combinations, physically separated by a Polyethylene terephthalate (PET) track-etched membrane inserts (23 mm) of 0.4 µm pore size (Falcon) in six-well plates, creating an upper and lower culture chambers. In the lower chamber, we inoculated the plasmid-free strain in 3.2mL of LB, and in the upper chamber, the plasmid-bearing strain in 2.5mL of LB. We incubated the co-cultures at 37ºC with constant agitation (130 rpm). We allowed the bacteria to grow in co-culture for 24 hours (reaching the stationary phase). Samples of the co-cultures were then retrieved, appropriately diluted and plated on selective media, as described for the mixed co-cultures.

4.3.5 Data analysis

We calculated the relative fitness (W_s) of the sensitive strain in each combination of conditions, both for ampicillin and ampicillin-free co-cultures, using the following equation (Bouma and Lenski 1988):

$$W_s = \frac{Log\ F_{final}/Log\ F_{inicial}}{Log\ B_{final}/Log\ B_{inicial}}$$

where F is the population of plasmid-free strain, and B the population of plasmid-bearing strain, calculated in CFU ml $^{-1}$, at the beginning and end of the experiments.

The relative fitness of sensitive cells in presence of ampicillin was normalized with the relative fitness of sensitive cells in absence of ampicillin, calculating the ratio:

$$ratio = W_s(with\ amp)/W_s(without\ amp)$$

The calculation of this ratio allowed us to discount potential pre-existing interference detected in the relative fitness of sensitive cells resulting from the co-

cultures without ampicillin. A negative value of ratio means that sensitive cells survived but their numbers diminished overtime. If bacterial replication is faster than bacterial death, the ratio becomes positive, implying that the number of sensitive cells is higher at the end than initially.

4.4 RESULTS AND DISCUSSION

4.4.1 Basal relative fitness of Plasmid-free cells

We started by measuring the effect that three conjugative plasmids of natural origin (R1, R16a and RP4) have on the fitness of *Escherichia coli* K12 MG1655 Δara Val^R by competing each of these strains with the parental strain plasmid-free *Escherichia coli* K12 MG1655 for 24 hours with no antibiotics (Figure. 1A). These measurements were performed in six conditions: two initial total cell densities (10^7 and 10^5 cell ml^{-1}); and three initial cell frequencies (plasmid-free:plasmid-bearing ratios as 1:99, 50:50 and 99:1). These six ecological conditions with each of the three plasmid-bearing cells comprise 18 different cases.

In Figure.S1A we show the values of Ws, i.e., of the relative fitness of the sensitive cells (those with the chromosomal markers *ara*+ – see methods). In this figure, one can see that, in seven cases, the plasmid imposed a cost on the plasmid-bearing strain and in four cases the plasmid-free strain had a lower fitness than the plasmid-bearing strains. In the other seven cases, the presence of the plasmids did not impose a significant effect on the fitness of their bacterial host.

4.4.2 Basal relative fitness of Plasmid-free cells when growing physically separated from plasmid-bearing cells

We then performed similar competitions, this time physically separating plasmid-free cells from plasmid-bearing cells with membranes with 0.4 µm pore size (Figure. 1B). With these membranes, direct contact and bacterial conjugation between cells of the two populations is blocked but there is exchange of culture media and free metabolites between the two strains. Figure.S1B shows the values of Ws when the membrane separates the two populations.

Independent-samples t-tests were conducted to compare the relative fitness of the plasmid-free strain when the strains were cultured physically mixed and when they

were physically separated, for each of the 18 cases (three plasmid-bearing strains, two densities and three frequencies). We observed (Figure. 2A) a significant difference between the two growth conditions in 12 of the conditions (Student T-test, $p<0.05$). In eight conditions, the relative fitness of the plasmid-free strain is higher when they are physically separated from the plasmid-bearing strains than when the two populations are mixed, and, in the other four conditions, the relative fitness of the plasmid-free strain decreased (Figure. 2A).

4.4.3 Survival of Sensitive strain in Ampicillin-enriched environment

Each plasmid of this study codes for β-lactamases, enzymes that destroy ampicillin and many other of the so-called β-lactam antibiotic class. *E. coli* cells harbouring these plasmids are able to grow in the presence of clinically relevant dosage of ampicillin (100μg ml^{-1}). The parental strain does not code for β-lactamases: when incubated alone in ampicillin-enriched media for 24 hours and then plated in ampicillin-free minimal media for 48 hours we observed no formation of colonies.

We co-cultured the two strains (plasmid-bearing and plasmid-free strains) physically mixed in the same 18 conditions mentioned in the two previous sections, only this time the growth media was supplemented with ampicillin (100μg ml^{-1}) (Figure. 1C). In these conditions, the sensitive strain was expected to be able to survive at some level, through the exploitation of the β-lactamase produced by the plasmid-bearing ampicillin-resistant strains (Domingues et al *submitted*) - indeed, we observed that the sensitive strain was able to survive in 15 out of 18 cases (Figure.S1C). Interestingly, in six of these cases, the frequency of sensitive cells increased along the competition (positive Ws values in Figure.S1C) and, in two of them (both involving the RP4 plasmid and competitions at high initial cell density) the fitness of sensitive cells was even higher than that of resistant cells (Ws values above 1 in Figure. S1C).

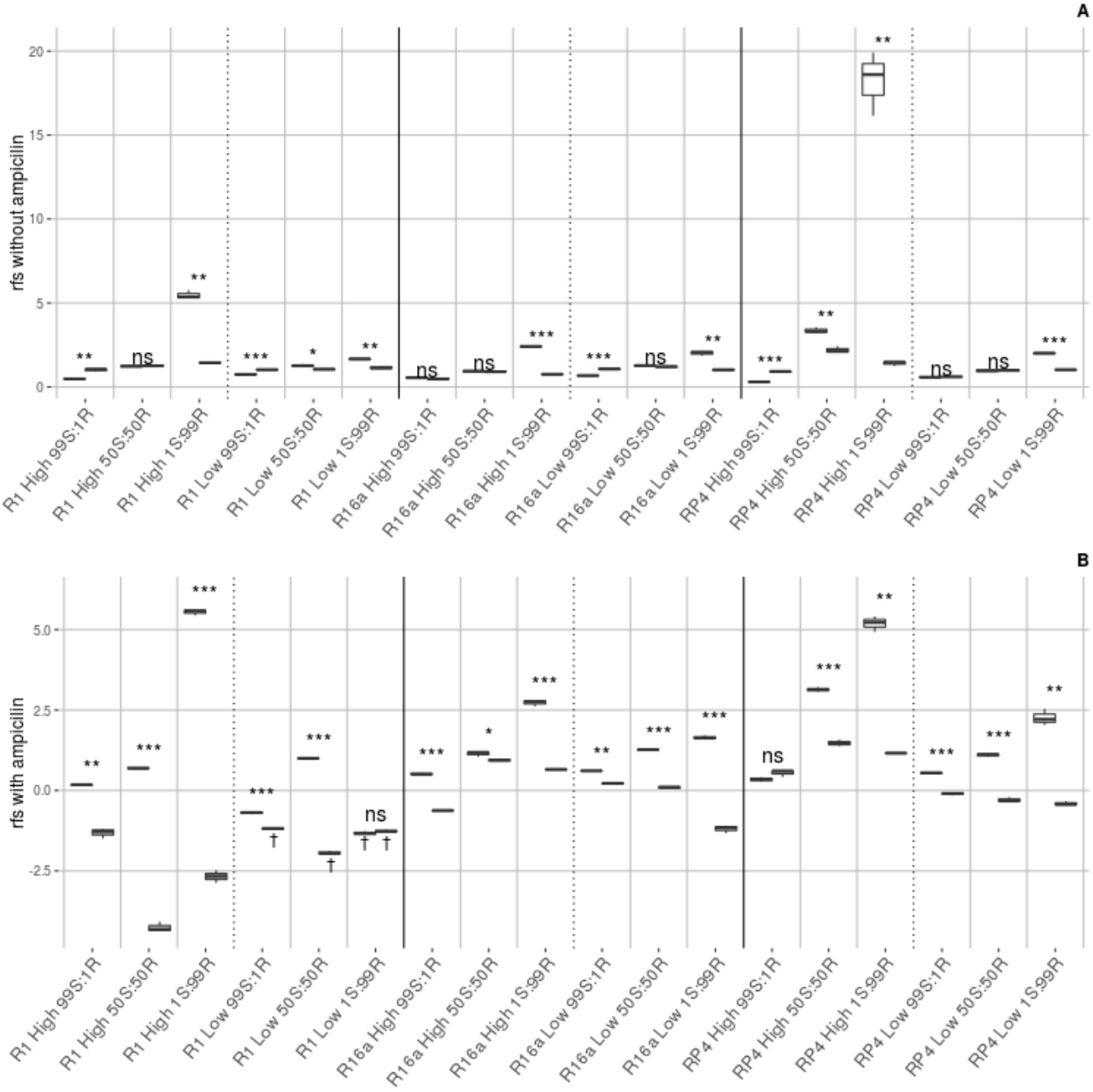


Figure 2. Comparison between the relative fitness of Plasmid-free cells (Ws), when physically mixed and separated. The plasmid-free strain was co-cultured with each one of the plasmid-bearing strains (containing one of three plasmids: R1, R16a, RP4), at 37ºC during 24 hours. All experiments were performed in triplicate. Bacteria were co-cultured in a combination of two different initial cell densities, three different initial cell frequencies and either physically mixed or separated, resulting in 36 different combinations of conditions (n=108), for each graphic. Independent-samples t-tests were conducted to compare the relative fitness of the plasmid-free strain when grown physically mixed (right Ws values) and physically separated by membranes of 0.4 µm pore size (left Ws values), either **(A)** without ampicillin, or **(B)** with ampicillin. Ws values can either be not significantly different (ns in the graphic) or they can be significantly different (* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$). R1, R16a and RP4 stand for each of the plasmid-bearing strains carrying each one of the plasmids. High stands for high initial cell density and Low for low initial cell density.99S:1R, 50S:50R, and 1S:99R refer to the initial cell frequency, in which S (ampicillin-sensitive) stands for the plasmid-free strain proportion, and R (ampicillin-resistant) stands for the plasmid-bearing strain proportion. † refer to cases of ampicillin-sensitive cell's death.

4.4.4 Survival of the Sensitive strain in Ampicillin-enriched environment when growing physically separated from resistant cells

As in the previous section, we performed competition experiments with the two strains (plasmid-bearing and plasmid-free strains) in the same 18 conditions with ampicillin, only this time physically separating plasmid-free cells from plasmid-bearing cells with membranes of 0.4 μm pore size (hence blocking direct contact and bacterial conjugation between cells of the two populations but allowing the exchange of culture media and free metabolites) (Figure. 1D and S1D).

Again, independent-samples t-tests were conducted to compare the relative fitness of the plasmid-free strain when the strains were cultured physically mixed and when they were physically separated, for each of the 18 combination of conditions. In 16 (out of 18) cases, the fitness of the sensitive cells is higher when physically separated from the plasmid-bearing cells (compared with the situation in which the two strains were mixed) (Student t-test $p < 0.05$) (Figure.2B). Within these results, there are some very interesting cases. For example, two initial frequencies at high density, sensitive cells were not surviving (in presence of ampicillin) when mixed with R1-bearing cells; however, when separated from the R1-bearing cells by the membrane, sensitive cells were able, not only to survive, but also to grow despite the presence of ampicillin (Figure.2B). In the other two cases, the fitness with or without the membrane was similar (Student t-test $p > 0.05$; Figure. 2B).

4.4.5 Final frequency of transconjugant cells in mixed co-cultures

In all previous sections we calculated the relative fitness of the plasmid-free strain (Ws) taking into account chromosomal markers (*ara*+ – see methods). However, when plasmid-bearing and plasmid-free cells are mixed (no membrane separating them), bacterial conjugation between plasmid-bearing and plasmid-free cells may occur and thus some plasmid-free cells become carriers of the plasmid. Bacterial conjugation, of course, may occur in presence or absence of ampicillin. Cells that just received a plasmid

or their descendants – the so-called transconjugants – may or may not represent an important part of the cells with the marker of the initially plasmid-free cells.

We recalculated the relative fitness of the plasmid-free strain, this time, subtracting the number of transconjugant cells present on the final population. If the number of transconjugants in the final population was high, the new relative fitness should become lower than the original relative fitness of the plasmid-free strain.

We observed 11 of such cases where the final percentage of transconjugant cells present in the final population was high enough to significantly decrease the relative fitness of sensitive cells ($p<0.05$; Independent-samples t-tests) (Table S1): eight cases with ampicillin and three cases without ampicillin.

Interestingly, 10 of these 11 cases (all the eight cases with ampicillin and two of the three cases without ampicillin) correspond to cases where Ws was higher when the two populations were physically separated from the plasmid-bearing population than when mixed. This suggests that bacterial conjugation and consequent gain of a plasmid was harming the plasmid-free strain, lowering its fitness.

Recall from last section that, with ampicillin, we observed eight more cases in which Ws is higher when the two populations are physically separated than when they are mixed. However, in these cases the final percentage of transconjugants was low (Table S1), hence not changing Ws whether or not taking into account the formation of transconjugants. Therefore, in these cases, plasmid transfer does not explain the fact that plasmid-free cells survive or grow more efficiently when separated from plasmid-bearing cells than when mixed with them. Rather, plasmid-free cells were somehow impaired by being cultured physically together with the plasmid-bearing strain, even if that conjoint growth did not result in a significant final population of transconjugant cells.

4.4.6 Ratio

Previous sections have shown that, on the one hand, plasmid-free cells often exploit β-lactamase produced by plasmid-bearing cells, and on the other hand, plasmid-bearing cells somehow manage to harm plasmid-free cells (either by transferring the plasmid or by another unidentified process involving direct cell contact).

If one calculates the ratio of Ws between the situation in which there is ampicillin and in which there is no ampicillin, we are removing the growth rate differences between the two bacterial populations, hence focusing on the ability of plasmid-free cells to endure and take advantage of β-lactamase produced by plasmid-bearing cells. These ratios can be calculated for the two experimental setups in which the two populations are separated or mixed. By calculating these two values of Ws ratios and then comparing them, we are assessing the effect of inhibiting physical contact between cells of the two populations to the fitness of sensitive cells when ampicillin is present, while discounting the intrinsic growth rate differences between the two bacterial populations (i.e. in the absence of ampicillin).

To compare the two ratios, independent-samples t-tests were conducted for each of the 18 conditions.

Surprisingly, we observed 17/18 cases (94%) in which the ratio is higher when the populations are separated than when populations are mixed (Student t-test $p<0.05$) (Figure. 3). This result corroborates results of previous sub-sections that plasmid-bearing cells harm other cells. Also interesting is the exception (1 case out of 18 cases): a case where the fitness of plasmid-free is higher when physically separated from the plasmid-bearing population than when together, but where that difference is even higher without ampicillin than with ampicillin.

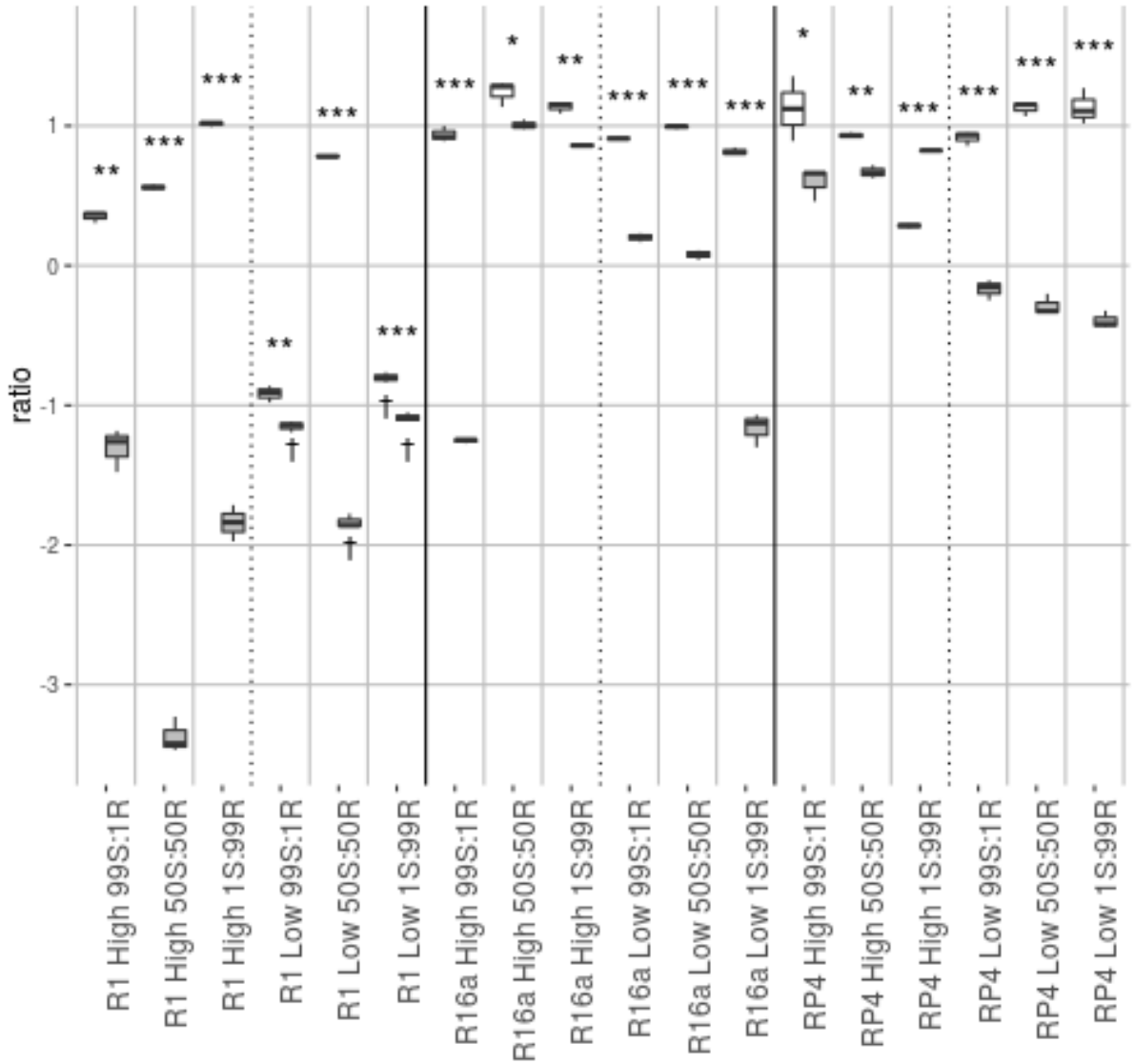


Figure 3. Comparison between the ratios of the relative fitness of Plasmid-free cells (rfs) , when physically mixed and separated. *The plasmid-free strain was co-cultured with each one of the plasmid-bearing strains (containing one of three plasmids: R1, R16a, RP4), at 37ºC during 24 hours. All experiments were performed in triplicate. Bacteria were co-cultured in a combination of two different initial cell densities, three different initial cell frequencies, either physically mixed or separated, and either with or without ampicillin, resulting in 74 different combinations of conditions (n=222). The ratio values were obtained through the division of rfs values with ampicillin by the rfs values without ampicillin. Independent-samples t-tests were conducted to compare the ratios when strains were grown physically mixed (right ratio values) and physically separated by membranes of 0.4 µm pore size (left rfs values), either. Ratios can either be not significantly different (ns in the graphic) or they can be significantly different (* for p<0.05, ** for p<0.01, and *** for p<0.001). R1, R16a and RP4 stand for each of the plasmid-bearing strains carrying each one of the plasmids. High stands for high initial cell density and Low for low initial cell density.99S:1R, 50S:50R, and 1S:99R refer to the initial cell frequency, in which S (ampicillin-sensitive) stands for the plasmid-free strain proportion, and R (ampicillin-resistant) stands for the plasmid-bearing strain proportion. † refer to cases of ampicillin-sensitive cell's death.*

4.5 CONCLUSION

Several mechanisms have been proposed to explain plasmid maintenance among bacterial populations. To our knowledge, this is the first report demonstrating that bacteria may use conjugative plasmids as harming agents. This corroborates previous observations (Oliver, Mcdougald et al. 1995, Hausner and Wuertz 1999) where plasmids induced a VBNC state to transconjugants. However, this observation revealed to be more complex than we anticipated, given that this may happen even when a low percentage of transconjugant cells was observed in mixed co-cultures. In other words, the plasmid does not need to transfer to recipient cells for the harming effect to occur.

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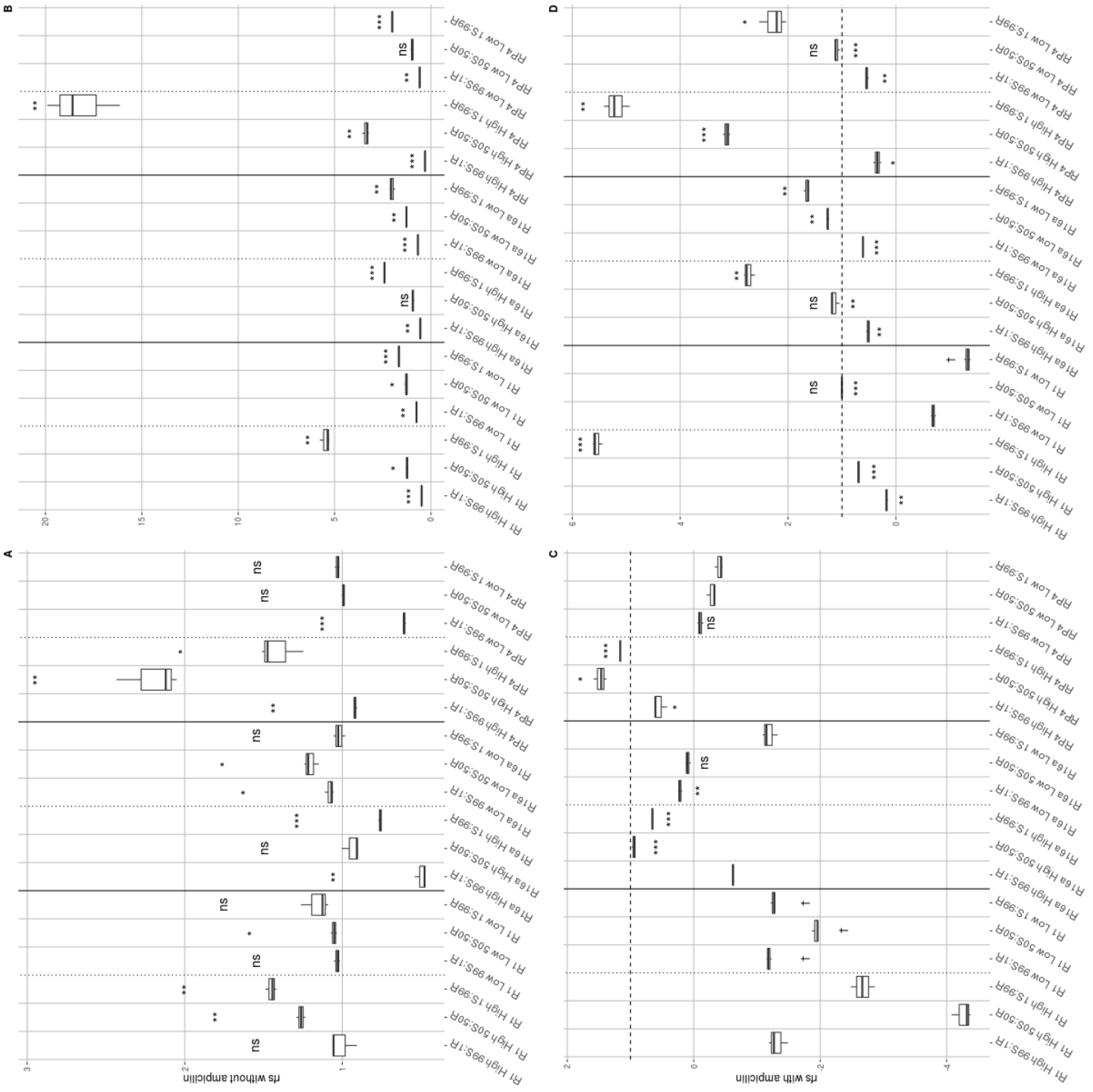

Figure S1. Relative fitness of Plasmid-free cells (rfs). The plasmid-free strain was co-cultured with each one of the plasmid-bearing strains (containing one of three plasmids: R1, R16a, RP4), at 37ºC during 24 hours. Co-cultures were conducted **(A)** physically mixed, without ampicillin; **(B)** physically separated (with membranes of 0.4 µm pore size), without ampicillin; **(C)** physically mixed, with ampicillin; and **(D)** physically separated, with ampicillin. All experiments were performed in triplicate. Bacteria were co-cultured in a combination of two different initial cell densities and three different initial cell frequencies, resulting in 18 different combinations of conditions (n=54), for each of the four different graphics. Independent-samples t-tests were conducted to properly evaluate the biological significance of the results. Positive fitness values (rfs > 0) correspond to the ability of plasmid-free cells to increase in frequency, hence, t-test results in which fitness values are significantly higher than 0 are presented below the rfs bars (in C and D). Fitness values superior to 1 imply the ability of plasmid-free cells to outgrow the plasmid-bearing cells, hence, t-test results in which fitness values are significantly higher than 1 are presented above the rfs bars (for A, B, C and D). Both t-test results are presented as follow: ns for not significantly different, * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$. R1, R16a and RP4 stand for each of the plasmid-bearing strains carrying each one of the plasmids. High stands for high initial cell density and Low for low initial cell density.99S:1R, 50S:50R, and 1S:99R refer to the initial cell frequency, in which S (ampicillin-sensitive) stands for the plasmid-free strain proportion, and R (ampicillin-resistant) stands for the plasmid-bearing strain proportion. † refer to cases of ampicillin-sensitive cell's death.

Table S1. Percentage of transconjugant cells in the final population. The values of percentages presented are the average for each condition. 2xStdErr stands for two times the standar error associated with each average value. Independent t test results refer to the comparison between the rfs with or without the final percentage of transconjugants included (values of rfs not shown). rfs values can either be not significantly different (ns in the table) or they can be significantly different (* for p<0.05, ** for p<0.01, and *** for p<0.001). R1, R16a and RP4 stand for each of the plasmid-bearing strains carrying each one of the plasmids. High stands for high initial cell density and Low for low initial cell density.99S:1R, 50S:50R, and 1S:99R refer to the initial cell frequency, in which S (ampicillin-sensitive) stands for the plasmid-free strain proportion, and R (ampicillin-resistant) stands for the plasmid-bearing strain proportion.

PLASMID	DENSITY	FREQUENCY	WITHOUTAMP (%)	2XSTDERR	T-TEST	WITHAMP (%)	2XSTDERR	T-TEST
R1	High	99S:1R	0,07	0,06	Ns	40,54	12,09	ns
R1	High	50S:50R	0,14	0,03	Ns	100,00	0,00	***
R1	High	1S:99R	2,03	1,45	Ns	85,47	13,51	*
R1	Low	99S:1R	0,01	0,00	Ns	0,00	0,00	ns
R1	Low	50S:50R	0,50	0,43	Ns	0,00	0,00	ns
R1	Low	1S:99R	0,10	0,02	Ns	0,00	0,00	ns
R16A	High	99S:1R	1,19	0,50	Ns	100,00	0,00	***
R16A	High	50S:50R	0,25	0,12	Ns	0,12	0,04	ns
R16A	High	1S:99R	14,48	4,79	*	48,90	19,58	*
R16A	Low	99S:1R	0,00	0,00	Ns	31,60	21,77	ns
R16A	Low	50S:50R	14,86	5,90	Ns	35,36	6,94	ns
R16A	Low	1S:99R	98,72	2,56	***	88,89	22,22	*
RP4	High	99S:1R	0,16	0,15	Ns	0,59	0,81	ns
RP4	High	50S:50R	2,33	1,13	Ns	0,61	0,19	ns
RP4	High	1S:99R	2,29	0,99	Ns	1,89	0,10	ns
RP4	Low	99S:1R	2,88	1,07	Ns	87,09	17,62	**
RP4	Low	50S:50R	100,00	0,00	***	86,82	26,36	**
RP4	Low	1S:99R	1,18	0,09	Ns	79,35	20,07	*

CHAPTER V:

5 THE SURVIVAL OF THE LUCKIEST OR THE INOCULUM EFFECT – EXPLAINING THE SURVIVAL OF SUSCEPTIBLE BACTERIA IN THE PRESENCE OF ANTIBIOTICS

Manuscript in preparation

Iolanda Domingues, João Gama, Diana Adão, Francisco Dionísio

5.1 ABSTRACT

Some enzymes confer antibiotic resistance by inactivating antibiotic molecules. This is the case, for example, of β-lactamases, enzymes that neutralise β-lactam antibiotics such as penicillin or ampicillin by hydrolysing the β-lactam ring of these molecules. Sometimes detoxification is so effective that nearby susceptible cells are able to survive and grow. With experiments involving *Escherichia coli* bacterial cells growing in the presence of ampicillin and a simple theoretical model, we show that the probability of survival of susceptible cells is proportional to the frequency of β-lactamase producer cells (as expected), but proportional to, at least, the sixth power of the frequency of susceptible cells. The biological meaning is that, for a susceptible cell to survive, at least six of them have to be near the β-lactamase producer. We argue that, while ampicillin is still not destroyed by β-lactamase, an significant fraction of antibiotic molecules attach to the cellular structures of susceptible cells. As such, these susceptible cells partially detoxify the environment. This gives the opportunity to a few, fortunate, susceptible cells to survive. As such, killed cells end up having an altruistic death. Meanwhile, β-lactamase completes detoxification, allowing the growth of the surviving susceptible cell. The clinical implications of this finding are worrisome, because it means that both resistant and sensitive bacteria, including non-pathogenic cells living in the same habitat, may collaborate to rescue susceptible pathogenic cells.

5.2 INTRODUCTION

Some bacterial cells resist to antibiotics by inactivating or modifying the antibiotic molecules. With this process, resistant cells eventually decrease antibiotic concentrations in the environment (for a review, see (Wright 2005)), allowing the survival of susceptible cells (Domingues, Gama et al. submitted). In principle, survival of susceptible cells is possible if these cells are fortunate to be growing near resistant cells that detoxifies the environment.

In a previous work we performed (Domingues, Gama et al. submitted) competition experiments between *E. coli* cells that produce β-lactamase (hence resistant to β-lactam antibiotics) and susceptible cells. These experiments were performed in the presence of ampicillin in order to understand the role of different resistant strains, habitat structuring, frequency of each strain, and total cell density, to the fitness of susceptible cells. At lower densities and when competition experiments were performed in a Petri dish, we observed a strange relationship between survival of susceptible cells and their frequency in the beginning of the competition experiments: in many cases, no susceptible cells survived at intermediate frequencies (about 50:50) but survived when susceptible cells were very common and producers of β-lactamase very rare (99:1). If the crucial condition for the survival of a susceptible cell is its proximity to a β-lactamase producing cell, a simple mathematical reasoning tells us that survival is more likely at 50:50 frequency than at 99:1 (see Results section). Our aim is to understand this phenomenon.

Of course, survival of susceptible cells should also depend on the speed to which resistant cells detoxify the environment. Apparently, (i) either susceptible cells have to undergo a period of transient antibiotic tolerance – resuming growth only after the concentration of the antibiotic decrease to values below the minimal inhibitory concentration (MIC) – or, (ii) resistant cells are very efficient at detoxifying the environment around themselves including nearby susceptible cells. The transient period of antibiotic tolerance (called persistence (Bigger 1944)) was already demonstrated to be important in rescuing susceptible cells when in presence of β-lactam antibiotics (Bigger 1944, Medaney, Dimitriu et al. 2016). These antibiotics are lethal only during the

process of cell division, so it is possible that persistence is caused by cell dormancy, a state in which cells are non-dividing. As such, susceptible cells survive during the period of dormancy – eventually initiating cellular division after detoxification. The second condition (very efficient detoxification by resistant cells) has been implicitly assumed by some authors to explain survival of susceptible cells. However, it could be argued that this second mechanism is unlikely because resistant cells would have to be extremely fast in the process of detoxification – in particular if the antibiotic concentration is tens or a hundred fold higher than the MIC.

For some reason, antibiotic efficacy decreases when cell density increases, namely lowering killing rates and increasing the MIC of the antibiotic (Udekwu, Parrish et al. 2009). The molecular mechanism of this phenomenon – called "inoculum effect" – is still not fully understood. Time-kill experiments have shown that the effect may be caused by a reduction in the effective concentration of the antibiotic in the medium by binding of the antibiotic to the cell structures of killed as well as viable bacteria (Udekwu, Parrish et al. 2009). So inoculum effect can be considered a mechanism of survival of susceptible cells.

Both persistence (Bigger 1944, Medaney, Dimitriu et al. 2016) and the inoculum effect (this paper) could explain the initial survival of susceptible cells. Then, they would be able to stay alive or even grow because they are close to cells capable of detoxifying the environment. Using ampicillin, *E. coli* susceptible cells, and β-lactamase producer *E. coli* cells, we study the role of each of these two mechanisms in the survival of susceptible cells.

5.3 MATERIALS & METHODS

5.3.1 Bacterial Strains

In this work we used two isogenic strains of *Escherichia coli,* the wild-type *E. coli* K12 MG1655 and *E. coli* K12 MG1655 *Δara* val^R. The latter strain harbours two chromosomal markers: *Δara* – deletion of the arabinose operon which disables the metabolism of arabinose monosaccharide; and val^R – mutation that enables the growth in the presence of valine (40 μg L^{-1}) in the absence of when isoleucine.

The conjugative plasmids R1, R16a and RP4, of natural origin, and the artificial plasmid pBR322 were inserted into *E. coli* K12 MG1655 *Δara* val^R, through bacterial conjugation (for the conjugative plasmids) or transformation (for the artificial and non-conjugative plasmid), resulting in four ampicillin resistant strains: *E. coli* K12 MG1655 *Δara* val^R (R1), *E. coli* K12 MG1655 *Δara* val^R (R16a), *E. coli* K12 MG1655 *Δara* val^R (RP4), and *E. coli* K12 MG1655 *Δara* val^R (pBR322). All four β-lactamase producing strains are identical (except for the different plasmids) and can therefore be mentioned solely according to the harboured plasmid. The wild-type strain will be mentioned henceforth as the ampicillin sensitive strain

5.3.2 Experimental conditions of co-culture

The sensitive strain was co-cultured with each of the ampicillin resistant strains, at low initial cell density (≈10^4 cells ml^{-1}) and at three initial cell frequencies – high frequency of sensitive cells (99S:1R), intermediate frequency of sensitive cells (50S:50R) and low frequency of sensitive cells (1S:99R), where S stands for sensitive and R for resistant.

We inoculated the sensitive and ampicillin-resistant strains, in each of the conditions, on petri-dishes containing solid LB medium (supplemented with 1.5 % agar) and incubated at 37ºC. Co-cultures were performed in media containing ampicillin (100 μg ml^{-1}). All co-cultures were performed in triplicate. We allowed the bacteria to grow in co-culture for 24 hours.

After the incubation period, the resulting bacterial culture was retrieved and suspended on 2 ml solution of $MgSO_4$ (0.01 M). Samples of the co-cultures were then retrieved, appropriately diluted and plated on selective media: M9 Minimal Medium with 3 mM $MgSO_4$, supplemented with 4 g L^{-1} of arabinose to select the sensitive strain; and supplemented with 4 g L^{-1} of glucose, 40 µg ml^{-1} of valine, and 100 µg ml^{-1} of ampicillin to select ampicillin resistant strains.

5.4 RESULTS AND DISCUSSION

5.4.1 Probabilistic models – relative proportion

Let's consider two *E. coli* populations spread on a Petri dish. Cells of one of the populations code for β-lactamase, conferring resistance to ampicillin, eventually detoxifying their vicinity. The other cells are ampicillin-susceptible. If the two populations are mixed in the Petri dish, susceptible cells may survive if they are close to a β-lactamase producing cell. If the proportion of susceptible cells is x, the proportion of β-lactamase producers is 1-x and so the probability that a susceptible cell is close to a β-lactamase cell is proportional to x.(1-x). The proportionality constant and hence the expected value of susceptible cells that survive in this process depends on several factors, such as cell density or diffusion speed of β-lactamase in the Petri-dish. The function x.(1-x) is a simple quadratic function, symmetrical around x = 0.5, and having its only maximum at x = 0.5, which means that the probability of encounter of a susceptible cell and a β-lactamase cell reaches its maximum when they both represent half of the total bacterial population.

Being symmetrical around x = 0.5, the probability of encounter is the same when susceptible cells are 1% or 99% of the total population. The probability of encounter when x = 0.5 is more than 25-fold higher than when the proportion of susceptible cells is 1% or 99%.

5.4.2 Number of surviving susceptible cells despite the presence of antibiotic

In our previous paper (Domingues, Gama et al. submitted), we performed competition experiments in solid LB medium containing ampicillin by mixing different proportions of β-lactamase producer *E. coli* cells and susceptible *E. coli* cells across a range of densities. In that paper we were mainly focused at measuring the fitness of susceptible cells relative to that of β-lactamase producing cells. Given the presence of ampicillin, most susceptible cells die before detoxification occurred. Then, to calculate the relative fitness of susceptible cells, we measured the number of susceptible cells

after 24 hours. With that final number, however, one can also retrospectively calculate the number of susceptible cells when they stopped dying.

Let S(t) represent the number of susceptible cells along time t. Assuming exponential growth:

$S(t)=S(0)2^{G}$ (Equation 1)

where G is the number of generations. One can estimate the total number of generations as the $Log_2(Rf/Ri)$, where Rf and Ri are final and initial number of resistant cells, respectively. Therefore, Equation 1 becomes:

$Sf = Si \times Rf/Ri$

where Si = S(0) is the initial number of susceptible cells (after stop dying due to the ampicillin) and Sf is the number of susceptible cells after 24 hours. Accordingly, one can estimate S(0) as:

$Si = Sf \times Ri/Rf$ (Equation 2)

In our previous paper, resistant cells used in competition experiments were producing β-lactamase because they harboured a plasmid of natural origin – we used three different plasmids, RP4, R1 and R16a (Domingues, Gama et al. submitted). In this work we performed further experiments, this time using the artificial non-conjugative plasmid pBR322 which also code for β-lactamase.

In Table S1 we present all values for Si, Sf, Ri and Rf for the four plasmids and for the different densities.

The growth of resistant cells (from Ri to Rf) and the death dynamics until Si is achieved (that is, in the time period between bacterial plating and the moment where cells stop dying) are both exponential phenomena. Therefore, we used a Student T-Test to test for differences of Log(Si) between frequencies 1S:99R and 99S:1R, where our null hypothesis is that they are similar (because the probability of encountering cells of the other strain is identical):

$Log(Si_{1S:90R}) = Log(Si_{90S:1R})$ (Equation 3)

In the previous sub-section we argued that the Log(Si) should be similar in both frequencies (1S:99R and 99S:1R). We also showed that the Log(Si) at frequency 50S:50R and at frequency 99S:1R should be related according to:

$Log(Si_{50S:50R}) = Log(25 \times Si_{90S:1R})$ (Equation 4)

Contrary to our initial expectations (i.e., just taking into account the relative proportion of susceptible and producer cells in a simple probabilistic model, see previous subsection), one can see in Table S1 that, in all cases tested,

$Log(Si_{1S:90R}) < Log(Si_{90S:1R})$ (Equation 5)

$Log(Si_{50S:50R}) < Log(25 \times Si_{90S:1R})$ (Equation 6)

For all cases, $p<0.05$ (Student t-test).

In words, when total cell density is low (plasmids RP4, R16a and pBR322) or intermediate (R1 plasmid), we experimentally observed that susceptible cells do not survive when they are 1% of the population (observation of less than half a colony – see methods), but they do survive when they are 99% of the population (Figure 1). This implies that there is an additional effect besides the simple probability model of encounter of susceptible and resistant cells.

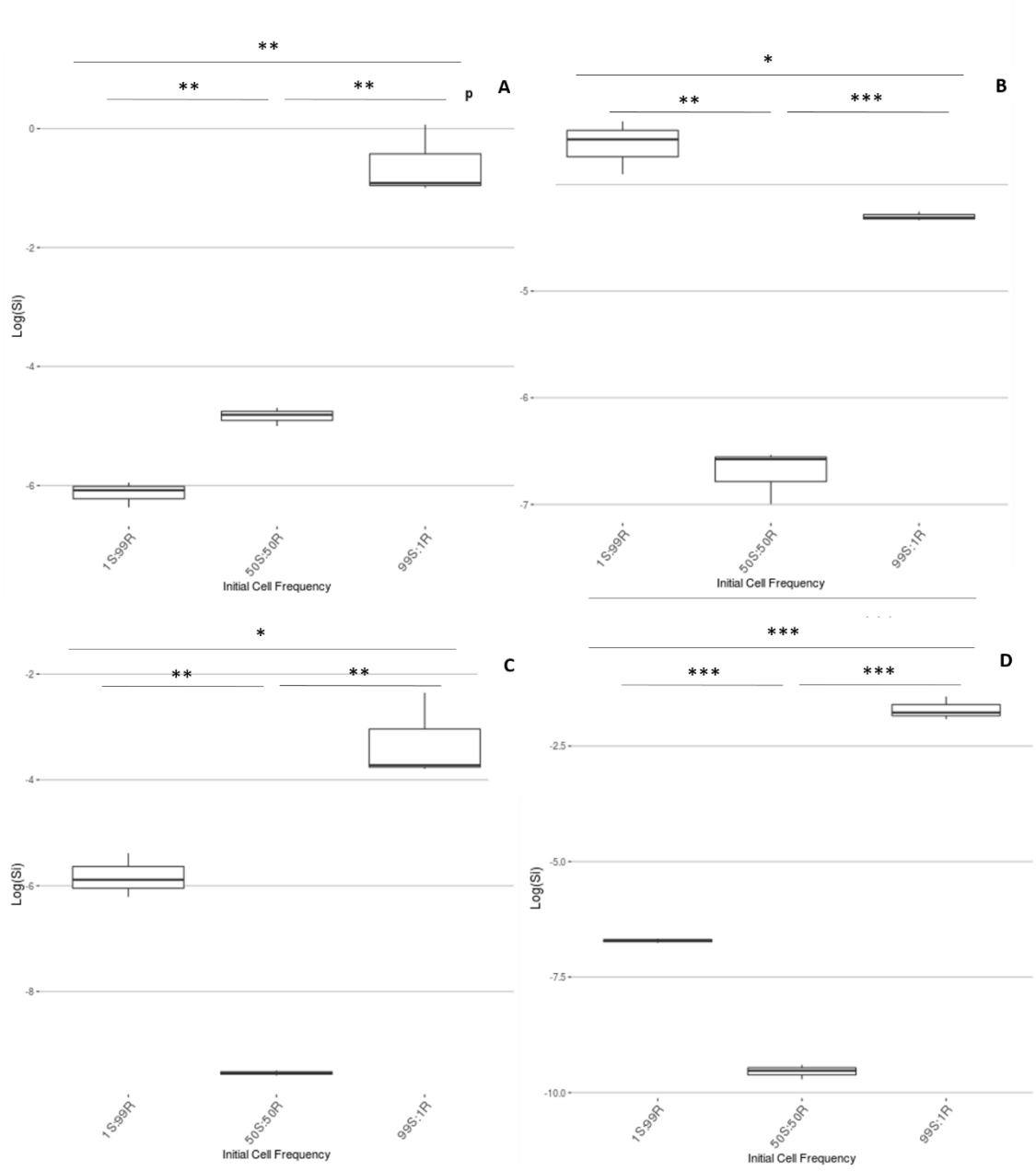

Figure 1 – Log(Si) values for each of the Plamsids. *Each graphic shows the log(Si) for each of the initial cell frequencies, for all strains used, carrying the plasmids:* ***(A)*** *pBR322;* ***(B)*** *R1;* ***(C)*** *R16a; and* ***(D)*** *RP4.. t-tests were used to determine if the log(Si) values are significantly diferents from each other. (* for p<0.05, ** for p<0.01, and *** for p<0.001).*

The probability of encounter when x = 0.5 is more than 25 fold higher than when the proportion of susceptible cells is 1% or 99%. Again, this simple mathematical fact does not fit with our experimental results: in several cases involving the four plasmids, one observed that no susceptible cells survived at x = 50%, but survived at x = 99%. Again, this implies that there is an additional effect besides the probability a susceptible cell grows near a resistant cell.

5.4.3 Probabilistic models – relative proportion and dormancy

To survive, susceptible cells have, somehow, to endure while detoxification occurs. This is possible, for example, if they are dormant, that is, not undergoing cell replication for a certain time period. Assuming that a proportion q of susceptible cells is dormant, the proportion of dormant cells is q.x. Therefore the probability of survival is proportional to q.x.(1-x). Again, the probability of surviving is the same if the proportion of susceptible cells is, say, 1% or 99% (because the product x.(1-x) gives the same result in both cases).

Again, and for the exactly the same reasons as before, this model does not explain our experimental results: (i) in all cases involving R16a, RP4 and pBR322 plasmids at lower cell densities or R1 plasmid at n intermediate density, susceptible cells survive when most cells are susceptible (99%) but not when only 1% of cells are susceptible; (ii) at 50S:50R we often observe no survival of susceptible cells but survival of susceptible cells at proportions 99S:1R, which is at odds with the model because q(1/2)(1-1/2) is about 25 times higher than q(0.99)(0.01).

5.4.4 Probabilistic models – altruistic death

As explained above, one should expect to observe more susceptible cells surviving when the proportion of susceptible and β-lactamase producer cells is 50S:50R than at 99S:1R or 1S:99R. Alternatively, one may envisage that the probability of survival is

proportional the probability that n susceptible cells grow near a β-lactamase producer cell: $x^n.(1-x)$, where n > 1 and x is the proportion of susceptible cells.

How much is n? According to our experimental data, there are some cases where susceptible cells survive when proportions are 99S:1R but not when proportions are 50S:50R. Therefore, n has to obey the condition:

$$\left(\frac{99}{100}\right)^n \cdot \frac{1}{100} > \left(\frac{1}{2}\right)^n \cdot \frac{1}{2}$$

Or n>5.7. So our experimental data dictates that n = 6 or higher.

According to this model, among six susceptible cells growing near a β-lactamase producer, at least one is expected to survive; however, with fewer susceptible cells near the β-lactamase producer, no susceptible cell survives. The inoculum effect may have a role in this process because, through binding of the antibiotic molecules to cell structures of killed cells, these cells are detoxifying the environment. As such, dying cells have an altruistic death. This process could explain survival of susceptible cells if there is no dormancy.

5.4.5 Probabilistic model – survival of the luckiest

Experiments were performed with *E. coli* cells, which are Gram-negative cells, and with the antibiotic ampicillin, which has it action at the murein layer at the inner cell membrane (becoming covalently attached to the PBP proteins of the murein). Therefore, before killing susceptible *E. coli* cells, ampicillin molecules have to cross their outer membrane. If cells are producing β-lactamase, and if these enzymes are mostly present in the space between the two cell membranes (as often is the case with Gram-negative cells), ampicillin molecules also have to cross the outer membrane; only then β-lactamase inactivates ampicillin molecules. The two processes turn both susceptible and resistant cells into sinks of ampicillin molecules. B-lactamase molecules may also cross the outer membrane and having its detoxifying effect outside cell. In any case,

therefore, both cell types (producers or non-producers of β-lactamase) should be responsible for the inoculum effect, and so it is not clear why susceptible cells have better conditions to survive at 99S:1R rather than 50S:50R or 1S:99R when total cells density is the same.

It is possible that the surviving (genetically) susceptible cells are the ones that, by chance, were not in a susceptible stage. If n = 6, this could mean that cells are susceptible to ampicillin about 5/6 of the time. This does not imply any mechanism of dormancy, just the existence of a short time period where cells are not susceptible to ampicillin. While the lucky cell is in the non-susceptible stage, the other cells (five susceptible and the β-lactamase producing one) are all detoxifying the local environment, although by means of very different mechanisms. This model – survival of the luckiest – explains why, at lower densities, survival of susceptible cells is sometimes observed at frequency 99S:1R but not 50S:50R nor 1S:99R.

5.5 CONCLUSION

The combination of our theoretical and experimental results indicate that two non-exclusive mechanisms explain the survival of sensitive cells. While producers of β-lactamase are still at the beginning of environmental detoxification, many antibiotic molecules attach to structures of sensitive cells. Most sensitive cells die because too many antibiotic molecules attached to their structures. Assuming antibiotic absorption is not a homogeneous process, it is expected that some cells live a bit longer than others. Meanwhile, producers of β-lactamase destroy many ampicillin molecules. Sensitive cells that were destined to die latter, may, after all, survive, thanks to the combined job of their close neighbours: β-lactamase producers and killed sensitive cells that absorbed big amount of drug molecules.

The second mechanism we propose is very similar, but the heterogeneity mentioned above has a biological cause: sensitive cells are in different phases of cell replication and one sixth of them are not sensitive to ampicillin for a while. Again these cells are destined to die as their neighbours, but they end up surviving because nearby resistant cells detoxify the local environment.

We have two altruistic populations in this process. B-lactamase producers, of course, are one of the altruistic populations: they help to the survival of some sensitive cells by producing big quantities of the detoxifying enzyme. However, killed sensitive cells have an altruistic death.

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5.7 SUPPLEMENTARY INFORMATION

Table S1. All log values for Si, Sf, Ri and Rf.

PLASMID	FREQUENCY	LOG(SI)	LOG(SF)	LOG(RI)	LOG(RF)
		-1,00	6,46	2,14	9,60
	99S:1R	0,07	7,31	2,07	9,32
		-0,92	6,83	2,11	9,86
		-4,70	2,49	4,14	9,93
PBR322	50S:50R	-4,81	2,48	4,07	9,96
		-5,00	2,08	4,11	9,79
		-6,37	-0,30	4,14	10,21
	1S:99R	-5,95	-0,30	4,07	9,72
		-6,08	-0,30	4,11	9,89
		-6,95	1,90	1,41	10,26
	99S:1R	-5,51	3,14	1,49	10,14
		-5,90	2,74	1,48	10,13
		-9,79	-0,30	2,00	10,09
PBR322	50S:50R	-9,95	-0,30	2,04	10,29
		-9,79	-0,30	2,18	10,27
		-7,34	-0,30	3,36	10,40
	1S:99R	-7,29	-0,30	3,37	10,36
		-6,57	-0,30	3,68	9,95
		-4,25	2,00	3,33	9,58
	99S:1R	-4,31	2,20	3,33	9,85
		-4,33	2,54	3,32	10,20
		-6,53	1,30	4,13	10,57
R1	50S:50R	-7,00	1,00	4,03	10,63
		-6,58	1,30	4,10	10,58
		-3,41	1,00	5,36	9,76
	1S:99R	-3,90	1,30	5,11	10,32
		-3,58	1,30	5,31	10,19
		-2,35	5,70	1,90	9,95
	99S:1R	-3,79	4,45	1,88	10,11
		-3,73	4,41	1,81	9,95
		-9,58	-0,30	2,54	10,42
R16A	50S:50R	-9,49	-0,30	2,60	10,39
		-9,54	-0,30	2,56	10,40
		-6,21	-0,30	3,90	9,81
	1S:99R	-5,39	-0,30	3,91	9,00
		-5,89	-0,30	3,94	9,53
		-1,92	6,60	1,30	9,82
	99S:1R	-1,43	6,62	1,68	9,73
		-1,77	6,34	1,74	9,86
		-9,53	-0,30	2,19	10,02
RP4	50S:50R	-9,71	-0,30	2,33	10,35

		-9,40	-0,30	2,29	9,99
		-6,76	-0,30	3,69	10,15
	1S:99R	-6,67	-0,30	3,74	10,11
		-6,71	-0,30	3,65	10,06

CHAPTER VI:

6 DISCUSSION

6.1 SOCIAL BEHAVIOUR AMONG BACTERIA: FROM HARMFUL BEHAVIOUR TO ALTRUISM

In this thesis (chapter II and IV), it was demonstrated that bacteria can use MGEs as allelopathic agents. Indeed, *E. coli* is able to use λ phage to lower the fitness of susceptible neighbouring bacteria (chapter II). Furthermore, and to our surprise, it was shown that *E. coli* is also able to use conjugative plasmids, of natural origin, to decrease the fitness of competing plasmid-free bacteria (chapter IV). The two results together, show that bacteria are able to use their MGEs as "biological weapons", against competing strains. In both cases, the behaviour can be classified as spiteful: the actor pays a cost (death, in the case of phage release, and the metabolic cost associated with conjugation, in the case of plasmid transfer) which results in a decrease in the fitness of competing strains (recipients).

Contrary to what happens for altruism, spite has to be preferentially directed towards non-related individuals (Hamilton 1970). But, how can an actor, specifically, identify one type of individuals to direct a behaviour towards? One possible answer, could be for the behaviour to have an associated "harming greenbeard" (Gardner and West 2004, Gardner and West 2010), that is, a way in which the actor could readily identify individuals that do not carry the gene responsible for the behaviour. According to that notion, the use of MGEs as spiteful agents, as described in chapter II and IV, can be classified as a "harming greenbeard". Bacteria related to the actor are immune to the infection by MGEs (this is true for both for phages and plasmids), while non-related individuals are not. Therefore, the spiteful behaviour is preferentially directed towards susceptible individuals, that are not immune to the MGEs, while kin individuals remains unharmed.

This "harming greenbeard" or type of kin discrimination, however, is not the only common characteristic between the use of phages and plasmids, as allelopathic agents. Contrary to other allelopathic agents (such as bacteriocins, or other toxins), phages can replicate within susceptible hosts, which leads to an amplification of the "toxin". This amplification can be beneficial to the producing strain, specially, when initially rare, because even if phages are few at first, they can rapidly increase in numbers (Brown, Le Chat et al. 2006). This ability to be an amplifiable allelopathic agent may, however, not

be exclusive of phages. The transfer of plasmids can also be amplified. Recipient cells that are freshly transformed in donor cells, that, is newly formed transconjugant cells, exhibit a high frequency of conjugation. This phenomenon is termed “epidemic spread” and can be a mechanism to ensure the rapid and complete conversion of a population into plasmid-bearing cells (Frost and Koraimann 2010). When plasmid behaves as spiteful agent, this “epidemic spread” phenomenon could have a similar outcome as phage amplification.

Differences between the amplification of plasmids and phages can be anticipated. Firstly, each donor cell can transfer the plasmid multiple times, while phages “donors” only release viral progeny once. Nonetheless, the quantity of phages released will be higher and, probably, affect more susceptible cells. Moreover, in the case of plasmids, the period of high frequency of conjugation is only transient, and restricted to newly formed transconjugant cells (Lundquist and Levin 1986, Frost and Koraimann 2010), while phage release is not restricted to a limited period or to recent recipients. After infecting a susceptible host cell, a phage can remain dormant (in a prophage state), during an indefinite period of time. Upon its reactivation, the phage will still be amplified, independently from the number of bacterial generations it remained dormant. Also, phage amplification appears to be a normal outcome, intrinsic to its nature, and probably observable with most (if not all) temperate phages. The same cannot be affirmed for plasmids. In fact, epidemic spread or high frequency of conjugation was only observed in few plasmids, while others did not present such transient behaviour and failed to invade through infectious transfer (Lundquist and Levin 1986). In the future, it would be interesting to, experimentally, determine the different dynamics of plasmid and phage amplification. Specially, since both plasmid and phage can also lead to the amplification and spreading of accessory genes (Frost and Koraimann 2010) and, particularly, because conjugation is still the main mechanism responsible for antibiotic resistance dissemination (Bennett 2008, Barlow 2009).

The hypothesis that bacteria could make a spiteful use of plasmids was, recently, raised by our group (Dionisio, Nogueira et al. 2012), and was, to our knowledge, experimentally demonstrated for the first time, in chapter IV. However, contrary to what happens in the case of the use of phages or bacteriocins as “biological weapons”,

mechanisms through which plasmids can harm competing bacterial strains is still unknow.

It was previously described, that only a small fraction of newly formed transconjugants could grow in selective media after conjugation within biofilms (Hausner and Wuertz 1999). However, in this case, the potential harming effect of plasmids was not considered. Instead, the authors hypothesised that the transconjugants became unable to grow because they were, somehow, in a viable but non-cultivable state or, alternatively, due to plasmid loss. This observation, however, corroborates the hypothesis that plasmids can be used as allelopathic agents.

One possible mechanism for plasmids to harm new recipient cells can be related with the initial cost a new bacterial host suffers, upon initial acquisition of the plasmid. Newly formed transconjugants divide more slowly during a few hundred generation (Bouma and Lenski 1988, Modi and Adams 1991, Dahlberg and Chao 2003, Dionisio, Conceicao et al. 2005). In this sense, the transfer of plasmid genes may harm new host directly.

Another possibility, is that the plasmid-free strain could be harmed by sex pili. The sex pili, or conjugative pili, is a mating structure that enables the transfer of plasmids between two bacteria (reviewed in (Cabezon, Ripoll-Rozada et al. 2015, Ilangovan, Connery et al. 2015)). High pili-mediated cellular attachment can result in death of recipient cells, due to destabilization of the outer membrane; a phenomenon referred to as “lethal zygosis” (Alfoldi, Jacob et al. 1957, Skurray and Reeves 1973, Garcillan-Barcia and de la Cruz 2008). In fact, lethal zygosis was, recently, hypothesised to be responsible for a frequency-positive advantage of plasmid carriers, when competing against a plasmid free strain (Dimitriu, Misevic et al. 2016). Interestingly, Dimitriu *et al.* (2016) even mention the possibility that plasmid carriage can lead to bacterial spiteful behaviour, although they do not discuss a possible relation between spite and lethal zygosis, nor any other specific mechanism of spite.

In the future, the mechanism through which plasmid-bearing strains harm plasmid-free strains should be further investigated. It would be interesting to determine whether plasmids can indeed harm bacterial cells directly, similarly to phages, or if the sex pili (or

other plasmid-related factor) can harm plasmid free cells. In which case, plasmid would carry an additional mechanism of spiteful behaviour (in addition to the carriage of bacteriocins), and would thus be able to, indirectly, harm bacteria not related to their bearer.

In this thesis, it was also demonstrated that different social contexts influence the survival of antibiotic-sensitive bacteria. It was shown that bacteria genetically sensitive to antibiotics can survive, and even grow, when in the vicinity of unrelated neighbours genetically resistant to antibiotics (chapter III and IV). Also, sensitive bacteria can rely on the presence of related sensitive bacteria to survive the initial impact of an antibiotic (chapter V). Together, this chapters show that depending on the initial demographic conditions, that characterize a bacterial population, different social interactions can be exploited for the survival of genetically sensitive bacteria.

For sensitive cells to survive in an antibiotic-enriched environment, the detoxification achieved by resistant neighbours should be enough to lower the antibiotic concentration to a bearable level. The celerity of this process can either allow the swift detoxification of the environment, and the consequent survival of sensitive strains, or it can be too slow to achieve a fast detoxification, leading to sensitive cell death. To achieve a fast detoxification, higher initial densities should be helpful, specifically, when it is associated with high initial frequency of resistant bacteria (higher production of β-lactamase). In this thesis (chapter III), it was indeed shown that, at higher initial cell density, survival of sensitive cells seemed to be more evident when sensitive cells were initially rare (negative frequency dependence) – which should translate into more initial production of β-lactamase. At higher initial cell density, more producer cells translate into a more rapid and efficient detoxification of the environment, leading to increased protection of sensitive cells. Also, the levels of survival of sensitive cells was higher when the resistant strain carried the plasmid RP4, which has been reported to express more β-lactamase than, for example, the plasmid R1 (Grinsted, Saunders et al. 1972, Uhlin and Nordstrom 1977, Crowlesmith and Howe 1980).

When the detoxification is too slow, however, the survival of sensitive cells can be impaired, or it can depend on additional mechanisms of survival. It was demonstrated

(chapter III) that at lower cell density protection was only observed when sensitive cells were initially abundant. Since this particular condition doesn't appear to maximize the production of β-lactamase, we suspected that another mechanism had to allow the initial survival of sensitive cells, namely persistence. Persistence is a condition in which a certain proportion of bacterial cells, among a replicating bacterial population, enter a physiologically dormant state, hence transiently displaying phenotypic tolerance to antibiotics. There are several mechanisms responsible for persistence, meaning that there might be different reasons why bacteria in a replicating population do not replicate, or replicate more slowly (Levin, Concepcion-Acevedo et al. 2014). Bacteria in such state of slowed replication can be refractory to antibiotics, and thus considered persisters. When sensitive cells first encounter an antibiotic, if some individuals in the population are in this kind of persister state, they could survive the initial action of the antibiotic, long enough to allow resistant cells to detoxify the environment. This initial mechanism of survival, could thus be preferentially observed in population with high initial frequency of sensitive cells.

Alternatively, or complementary to persistence, an inoculum effect could also explain why a few sensitive cells have to be near a producer cell for a sensitive cell to survive. It has been known, for some decades, that the degree of killing-ability of an antibiotic decrease with the size of the bacterial inoculum – a phenomenon known as the inoculum effect (Udekwu, Parrish et al. 2009, Bhagunde, Chang et al. 2010). The exact mechanism, through which the inoculum effect acts, is still not totally clear. One possibility is that when surrounding sensitive cells absorb antibiotic molecules and die, these dead cells contribute towards the detoxification of the environment (Udekwu, Parrish et al. 2009, Bhagunde, Chang et al. 2010). In the specific case of our experimental results (chapter V), when there is a high initial number of sensitive cells, some may survive to the early contact with the antibiotic simply due to the death of surrounding sensitive cells (Udekwu, Parrish et al. 2009, Bhagunde, Chang et al. 2010).

Both cases of initial survival, rely on the initial demographic condition (high frequency of sensitive cells, that allow initial survival) and social context (presence of genetically resistant bacteria that detoxify the environment). Therefore, when the detoxification of the environment takes longer to achieve, because of to the initial

conditions of bacterial growth, for example, due to lower initial density of β-lactamase producer cells, a dual social antibiotic resistance allows the survival of genetically sensitive bacteria. When β-lactamase is still in a low concentration, sensitive cells (when abundant) act as social shields against the antibiotic, allowing some of their relatives to survive, long enough for the second social resistance phenomenon to happen – the detoxification of the environment achieved by β-lactamase producer cells (when β-lactamase concentration reaches higher concentrations).

It has been known for decades that the success of antibiotherapy, during the course of a bacterial infection, depends on host associated factors, such as age, general health condition or genetical background (Sorensen, Nielsen et al. 1988). Now, in this thesis (chapter III and V), it is shown that the interactions between the initial demographic conditions of a bacterial population, as well as its social context, may also drastically affect the ability of an antibiotic to kill genetically sensitive bacteria. In this thesis, it was also demonstrated that, in the context of antibiotic resistance, bacteria can simultaneous exhibit different social behaviours, ranging from harmful to cooperative behaviours (chapters III, IV and V). More specifically plasmids carried by bacteria may act as multi-levelled drivers of social interactions, acting as allelopathic agents that decrease the growth rate of sensitive strains (chapter IV), but also as promotors and enforcers of the cooperative production of β-lactamase (chapters III, IV and V).

Bacteria carrying plasmids are susceptible to invasion by certain phages, called male-specific phages (Jalasvuori, Friman et al. 2011). These phages use sex pili to invade a bacterial cell, hence having a very different way of interacting with bacterial cells than that of the phage used in this thesis (chapter II). Bacterial cells not expressing sex pili are not susceptible to male-specific phages. In the future, it would be interesting to determine how the acquisition of a conjugative plasmid, followed by the infection of a male-specific phage, would affect the social resistance and general virulence of bacterial parasites. We anticipate that results would be more complex, but also more interesting. Suppose that a given bacterial cell carries a conjugative plasmid that codes for the β-lactamase enzyme. These cells may help neighbouring cells to survive to the presence of ampicillin (as shown in chapter III, IV and V). However, if male-specific-phages were added to the competition experiment, different outcomes can be predicted. For one,

the cost of carrying the plasmid would become much higher, because it would lead host cells to be attacked by phages. This cost could, then, select plasmids not expressing sex-pili, that might be present in the population, i.e., non-conjugative plasmids (that might not carry the β-lacatmase gene). Alternatively, plasmid, present in either donor cells or newly formed transconjugants, might, mutate and cease to express sex-pili, hence becoming resistant to male-specific phages, while still producing β-lactamase to the local community. Another possibility, would be for the β-lactamase genes to be recruited into the chromosome, and the remaining plasmid to be discarded by the bacterial cell. Which could eliminate the social behaviours promoted by the plasmid. Independently from the possible outcome, it would be interesting to access if adding phages would benefit or impair the social antibiotic resistance, as well as the virulence of bacteria. This information could shed some more light into the potential use of alternative therapies to treat bacterial infection, such as phage therapy (in this case, specifically targeting the main mechanism of antibiotic-resistance spread – bacterial conjugation).

This synchronization of multiple social behaviours, achieved by MGEs, can potentially lead to an increased virulence of bacteria, in the context of a host infection. It has been demonstrated, that high relatedness between bacteria, within an infected host, can select for higher levels of virulence factors (specifically, siderophores) – a form of cooperation that allow bacteria to better infect the host, leading to increased virulence (West and Buckling 2003). Likewise, the results presented in this thesis suggest that, not only cooperative behaviours, but also spiteful ones can promote an increase in relatedness of a bacterial population, potentially allowing for the selection of higher levels of virulence.

Since antibiotic resistance is one of the most clinically relevant problems of our era, it is of great importance to further elucidate the ways in which bacterial social behaviours can shape the outcome of antibiotherapy. The results presented in this thesis, were performed in laboratorial settings, and although the general conclusion might be the same in an actual animal infection, it would be interesting and important, in the future, to determine how the social context, specially the interaction of spiteful and altruistic behaviours, and the initial demographic conditions of a bacterial

population, can affect the virulence of bacteria and the outcome of a disease. In sum, it would be important that, in the future, when choosing or designing an antibiotherapy, to consider not only host related factors, but also bacterial factors, particularly, factors that might be coordinated through social interactions.

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"If you're not cheating, you're not trying!"

Eduardo "*Eddie*" Guerrero, Professional Wrestler